A study to determine if the concentration of phenobarbitone in serum can be accurately predicted from that measured in dried blood spot specimens.

A DISSERTATION SUBMITTED TO THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY, IN PARTIAL FULFILMENT OF THE REGULATIONS FOR THE AWARD OF M.D. DEGREE IN PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2015.

DEPARTMENT OF PHARMACOLOGY AND CLINICAL PHARMACOLOGY

CERTIFICATE

This is to certify that this dissertation entitled " A study to determine if the concentration of phenobarbitone in serum can be accurately predicted from that measured in dried blood spot specimens." submitted by Dr. Aswathy Rachel Thomas, in partial fulfillment of university regulations for the award of M.D. Pharmacology (Branch VI) Degree examination of The Tamil Nadu Dr. M.G.R. Medical University, Chennai is a bonafide original work done under my direct guidance and supervision and completed to my utmost satisfaction.

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DECLARATION

I, Dr. Aswathy Rachel Thomas, do hereby declare that this dissertation entitled *" A study to determine if the concentration of phenobarbitone in serum can be accurately predicted from that measured in dried blood spot specimens."* has been done by me under the direct guidance of Dr. Kalpana Ernest, Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, and Dr Denise Fleming, Honorary Professor, Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, in partial fulfillment of university regulations for the award of M.D. degree in Pharmacology (Branch VI). I have not submitted this dissertation in any part or full to any other university or towards any other degree.

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PHARMACOLOGY (BRANCH VI) EXAMINATION TO BE HELD IN APRIL 2015. DEPARTMENT OF PHARMACOLOGY AND CLINICAL PHARMACOLOGY ABSTRACT Background: It is well accepted that phenobarbitone (PHB) requires therapeutic drug monitoring (TDM). The conventional matrix of serum or plasma used for TDM of phenobarbitone is not easily, cheaply or safely transported long distances. An alternative is to measure phenobarbitone in dried blood spots (DBS). These are stable and easily transported. This provides an affordable and safe alternative for rural hospitals in India. Objectives: To determine if the concentration of phenobarbitone in serum can be accurately predicted either directly or with an equation from that in dried blood spots. Methods: Thirty six patients between (and including) the ages of 18 to 65, who were on phenobarbitone, were enrolled into the study after informed consent was obtained. The

DBS specimens were prepared by spotting 20µl **of whole blood** 28

on a standard Whatman filter paper using a calibrated micropipette. Separate validated HPLC assays were used to measure the concentration of phenobarbitone in serum and DBS. Normal distribution was assessed, precision and bias were calculated along with regression using the Spearman rank correlation

test and the Mann Whitney-U paired **test** was performed. Results: **The** 2

imprecision and the bias between the measured and the predicted serum concentrations, when corrected for haematocrit were 8% and 0.49% respectively. The data were not normally distributed. The correlation coefficient was 0.99 between the serum and corrected dried blood spot (CDBS) concentrations (Spearman rank correlation test). No statistical difference was found

between the two groups by the **Mann Whitney-U test (p=0.** 3919). Conclusion: **The** concentration **of** 35

phenobarbitone in serum can be accurately predicted directly from that measured in a DBS. This means that a simple, cheap, easily accessible dried blood spot TDM service is available for rural hospitals in India INTRODUCTION A large number of people around the world are affected by epilepsy. The burden of epilepsy is higher in the developing than developed countries. Upto 70% of the people respond to treatment if adequately treated. Phenobarbitone is the most widely prescribed antiepileptic drug worldwide because of its low cost, good efficacy and convenient dosing of once daily. Therapeutic drug monitoring is a common tool used to optimise drug therapy by individualising the dose of the drug. Antiepileptic drugs are commonly subjected to TDM due to inter-individual variability in its pharmacokinetics, numerous drug interactions being potent enzyme inducers or inhibitors

Medicine: An Update", American Journal of Hospice and Palliative Medicine, 2012.

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Abbreviations

S.No.	ABBREVIATION	FULL FORM
1.	ACN	Acetonitrile
2.	AMPA	α-Amino-3-hydroxy-methyl-4-isoxazole-propionic acid
3.	CDBS	Corrected Dried blood spot
4.	Concn	Concentration
5.	CYP	Cytochrome P
6.	GABA	Gamma Amino Butyric Acid
7.	GC	Gas liquid chromatography
8.	HPLC	High Performance Liquid Chromatography
9.	IBE	International Bureau for Epilepsy
10.	ILAE	International League Against Epilepsy
11.	IS	Internal Standard
12.	LC-MS/MS	Liquid chromatography tandem mass spectrometry
13.	LLOQ	Lower Limit of Quantification
14.	NMDA	N-methyl-D-aspartate
15.	PCV	Packed Cell Volume
16.	PHB	Phenobarbitone
17.	QC	Quality Control
18.	RPLC	Reversed Phase Chromatography
19.	RT	Retention time
20.	Std	Standard
21.	TDM	Therapeutic Drug Monitoring
22.	UDBS	Uncorrected Dried blood spot
23.	UGT	Uridine diphosphate glucuronosyltransferase
24.	DBS	Dried Blood spot

Table of Contents

Abstract

Background

It is well accepted that phenobarbitone (PHB) requires therapeutic drug monitoring (TDM). The conventional matrix of serum or plasma used for TDM of phenobarbitone is not easily, cheaply or safely transported long distances. An alternative is to measure phenobarbitone in dried blood spots (DBS). These are stable and easily transported. This provides an affordable and safe alternative for rural hospitals in India.

Objectives

To determine if the concentration of phenobarbitone in serum can be accurately predicted either directly or with an equation from that measured in dried blood spots.

Methods

Thirty six patients between (and including) the ages of 18 to 65, who were on phenobarbitone, were enrolled into the study after informed consent was obtained. The DBS specimens were prepared by spotting 20µl of whole blood on a standard Whatman filter paper using a calibrated micropipette. Separate validated High Performance Liquid Chromatography (HPLC) assays were used to measure the concentration of phenobarbitone in serum and DBS. Normal distribution was assessed, precision and bias were calculated along with regression using the Spearman rank correlation test and the Mann Whitney-U paired test was performed.

Results

The imprecision and the bias between the measured and the predicted serum concentrations, when corrected for haematocrit were 8% and 0.49% respectively. The data were not normally distributed. The correlation coefficient was 0.99 between the serum and corrected dried blood spot (CDBS) concentrations (Spearman rank correlation test). No statistical difference was found between the two groups by the Mann Whitney-U test (p=0.3919).

Conclusion

The concentration of phenobarbitone in serum can be accurately predicted directly from that measured in a DBS. This means that a simple, cheap, easily accessible dried blood spot TDM service is available for rural hospitals in India.

Keywords

Dried blood spot, phenobarbitone, serum, TDM

Introduction

A large number of people around the world are affected by epilepsy. The burden of epilepsy is higher in the developing than developed countries. Upto 70% of the people respond to treatment if adequately treated. Phenobarbitone is the most widely prescribed antiepileptic drug worldwide because of its low cost, good efficacy and convenient dosing of once daily.

Therapeutic drug monitoring is a common tool used to optimise drug therapy by individualising the dose of the drug. Antiepileptic drugs are commonly subjected to TDM due to inter-individual variability in its pharmacokinetics, numerous drug interactions being potent enzyme inducers or inhibitors and the non-linear relationship between the dose and the serum/plasma concentration of the drug. It is well accepted that phenobarbitone requires therapeutic drug monitoring.

The standard matrix for measuring drug concentration is serum/plasma. The conventional serum or plasma samples used to measure phenobarbitone pose two problems. The first is that, it cannot be easily transported, and in order to be sent from a rural hospital to a therapeutic drug monitoring facility would require cold chain conditions, thereby increasing the cost of transport. Secondly, the specimens being transported as whole blood, plasma or serum increase the risk of biohazard to handlers. A relatively newer technique which is becoming more common is the use of the dried blood spot. These specimens have the advantage of being stable and less infectious, and hence can be easily and cheaply transported by regular mail for analysis to a TDM facility.

This study was done to determine if the concentration of phenobarbitone in serum can be accurately predicted from a DBS specimen. This will open avenues for these specimens to be

used as a safer and cheaper alternative for rural hospitals to send blood for phenobarbitone monitoring.

Aim & Objectives

Aim

To determine if the concentration of phenobarbitone in serum can be accurately predicted from that measured in dried blood spot and to determine if any equation needs to be applied to the DBS result to obtain the serum concentration.

Objectives

1. To develop and validate a High Performance Liquid Chromatography (HPLC) assay to measure the concentration of phenobarbitone in serum.
2. To develop and validate a HPLC assay to measure the concentration of phenobarbitone in dried blood spot.
3. To measure the concentration of phenobarbitone in serum, using HPLC.
4. To measure the concentration of phenobarbitone in dried blood spot specimens obtained on standard Whatman filter paper, using HPLC.
5. To determine the correlation between phenobarbitone concentration in serum and DBS.
6. To determine if any equation needs to be applied to the dried blood spot result to obtain the serum concentration.
7. To determine the precision and the bias of the equation in predicting the serum concentration of phenobarbitone using an independent group of patients.

Review of literature

Burden of neurological disorders

Neurological disorders are an important cause of mortality and morbidity worldwide (1). Most of these disorders occur more commonly in the developing countries and many of them are both preventable and treatable (1). Epilepsy comes under the category of serious neurological disorders (2). There is a two to three times increased risk of dying prematurely in people with epilepsy than in a general population (3).

Burden of epilepsy

According to WHO, epilepsy accounts for 0.5% of the global disease burden (4). 50 million people around the world are affected by epilepsy (3).

With more than 85% of the people with epilepsy being present in the developing countries, epilepsy is much more common in the developing than the developed world (5). The seizures in upto 70% of epileptic patients can be completely controlled (3). Yet, in the developing countries, upto three fourth of the epileptic patients either do not receive any treatment or receive inadequate treatment (3). This is because of multiple social, economic and political barriers such as social stigma, limited availability of resources and the high cost of newer drugs (6).

There are not many epidemiological studies done on the burden of epilepsy in India. A recent meta-analysis showed an overall prevalence of 5.33 per 1000 (7). The number of Indian people with epilepsy was estimated to be 5.5 million in the year 2001 (7). The incidence was found to be 49.3 per 1,00,000 population (8,9), thus giving rise to half a million new cases every year. In India, the treatment gap in epileptic patients is found to be as high as 50-70% (10). The reasons for this include the high cost of drugs, misconceptions

about the disease leading to faulty practices, failure to identify patients with epilepsy and unavailability of treatment to patients (11).

Epilepsy

The International League Against Epilepsy (ILAE) and the International Bureau For Epilepsy (IBE) has defined epilepsy as " A disorder of the brain characterized by an enduring predisposition to develop epileptic seizures and by the neurobiologic, cognitive, psychological, and social consequences of this condition " (12). An epileptic seizure is characterised by abnormal excessive brain neuronal activity resulting in the transient clinical manifestations (12).

Etiological classification of epilepsy

Classification of both epilepsy and seizures are important for its proper management. Identification of the type of seizure is important for selecting the appropriate drug for that particular seizure as well as to focus on the diagnostics relevant to the possible aetiologies (13).

The International League Against Epilepsy has classified epilepsy on the basis of aetiology, into idiopathic, symptomatic, provoked and cryptogenic epilepsies (14). Idiopathic epilepsy is mainly genetic in origin and there is no gross abnormality, anatomically or pathologically (14). An example of idiopathic epilepsy is generalised epilepsy with febrile seizures (14). Symptomatic epilepsy can have a genetic or an acquired origin. There are gross abnormalities, anatomically and pathologically, and the person will have the signs and symptoms of the underlying disease condition which is causing the epilepsy (14). Examples of genetic or developmental causes resulting in symptomatic epilepsy are Down's syndrome

and Tuberous sclerosis (14). A few acquired causes include cerebral tumours, cerebral trauma, cerebral infections, cerebrovascular disorders and cerebral immunogenic conditions like systemic lupus erythematosus (14). In provoked epilepsy, the seizures are precipitated by some systemic or environmental factor like fever, drugs, alcohol or toxins (14). There are no gross neuro-abnormalities – anatomically or pathologically (14). Reflex epilepsies are a type of provoked epilepsy which is induced by factors like loud noise, light or hot water (14). Cryptogenic epilepsy is one in which the cause of the epilepsy is not known. It accounts for a minimum of 40% of all the epilepsies seen in the adult population (14).

Classification of seizures

The ILAE Commission on Classification and Terminology (2011-2013) provided an updated classification of seizures (15). Seizures were classified into generalised, focal and unknown seizures (15). Generalised seizures were further classified into tonic-clonic, myoclonic, absence, tonic, clonic and atonic seizures (15). While generalised seizures involve both the cerebral hemispheres, focal seizures typically involve only one cerebral hemisphere (13). However, focal seizures can evolve and become generalised in nature and the focus of such a seizure is usually in the frontal lobe (13). Unknown seizures are those which do not show enough features to classify them under either generalised or focal seizures (15).

Status epilepticus

Status epilepticus is a condition in which a single seizure lasts for a duration of more than 30 minutes or two or more seizures occur without attainment of consciousness in between (16). It is a commonly encountered medical emergency and is associated with high morbidity and

mortality (17). When status epilepticus does not respond to the standard treatment , it is said to be refractory in nature and is associated with even greater mortality (17).

A history of pre-existing epilepsy is commonly seen in these patients. In such patients, the mechanism for status epilepticus is a sharp fall in the serum concentration of antiepileptic drugs due to factors like noncompliance to treatment (17).

Mechanisms in the initiation and spread of seizure activity

There are two phases involved in this (13) :

a. Seizure initiation phase
b. Seizure propagation phase

Seizure initiation phase (13)

This phase starts with a burst of action potentials or repetitive action potentials. These action potentials are fired at a high frequency. The repetitive action potentials are due to prolonged depolarization of the neuronal membrane. Prolonged depolarization occurs due to the entry of sodium (Na^+) ions into the cell through the voltage gated sodium channels. This is followed by hyperpolarization caused by the action of the inhibitory neurotransmitter, ɣ-amino butyric acid (GABA) or through potassium (K^+) channels.

Seizure propagation phase (13)

The seizure activity is propagated by recruiting the surrounding neurons. The surrounding neurons get activated by the following mechanisms:

1) The extracellular potassium increases. This weakens the hyperpolarization and causes depolarization of the surrounding neurons.

2) In the presynaptic terminals, calcium increases. This increases the release of neurotransmitters.
3) The excitatory neurotransmitter, N-methyl-D-aspartate (NMDA) activates the NMDA receptor. This causes the inflow the calcium into the cell and activates the neurons.

When sufficient number of neurons is recruited from the surrounding area, the seizure activity will spread to other contagious areas.

Drugs used in the treatment of epilepsy

There are numerous drugs available for the treatment of epilepsy. Choosing the appropriate drug according to the seizure type is important.

According to the ILAE guidelines for the treatment of epileptic seizures, the choice of an antiepileptic drug should be individualised (18). In addition to the efficacy of the drug, the physician should also take into consideration other factors such as drug safety and tolerability, formulation, pharmacokinetics and cost of the drug while choosing a drug to treat a newly diagnosed or untreated epileptic patient (19).

Table 1 shows the numerous drugs used in the treatment of epilepsy with their mechanisms of action.

Table 1 : Antiepileptic drugs with their mechanism of action (20)

S.no.	Mechanism of action	Antiepileptic drug
1	Sodium Channel modulators, Stabilize the fast inactivated state	Phenytoin, Carbamazepine, Lamotrigine, Oxcarbazepine, Topiramate, Valproate
2	Sodium Channel modulators, Stabilize the slow inactivated state	Lacosamide
3	Calcium channel blockers, Block high voltage P/Q type channel	Gabapentin, Pregabalin
4	Calcium channel blockers, Block T type channel	Ethosuximide
5	$GABA_A$ receptor allosteric modulators	Benzodiazepines, Phenobarbitone, Topiramate, Carbamazepine, Oxcarbazepine
6	GABA transaminase inhibitors	Vigabatrin
7	NMDA receptor antagonists	Felbamate
8	AMPA/kainate receptor antagonists	Phenobarbitone, Topiramate
9	Enhancers of HCN channel activity	Lamotrigine
10	SV2A protein ligand	Levetiracetam
11	Brain carbonic anhydrase inhibitors	Acetazolamide, Topiramate, Zonisamide

Phenobarbitone

Introduction

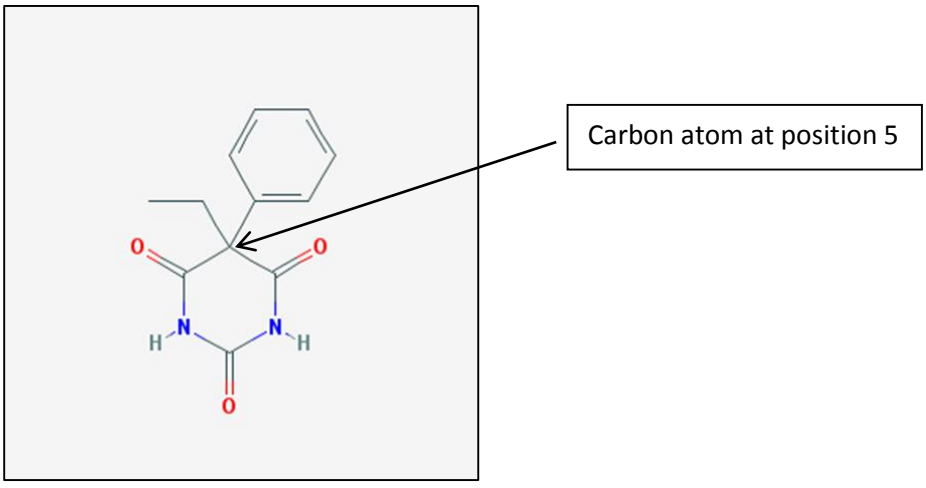
Chemically known as 5-ethyl-5-phenyl barbituric acid, phenobarbitone is one of the oldest anti-epileptic drugs which is still available in the market (21). It's anti-epileptic properties were accidentally discovered in the year 1912, when a young doctor named Alfred Hauptmann found that it decreased seizures when given as a sedative in a patient with epilepsy (21). Phenobarbitone is a long acting barbiturate and became the second drug in its class to be clinically used after barbital (21).

Being the most cost-effective anti-epileptic drug, makes phenobarbitone the most widely prescribed anti-epileptic drug in the developing world (21). Its cost-effective nature enables it to play an important role in reducing the treatment gap in the developing countries (21).

Structure

Phenobarbitone has a molecular weight of 232.23. It is denoted by the molecular formula $C_{12}H_{12}N_2O_3$ (22).

*Figure1:Structure of phenobarbitone (*Reference: http://pubchem.ncbi.nlm.nih.gov/summary)



Structure – activity relationship

Barbiturates have two substituents attached to the carbon atom at position 5 *(Figure 1)* (20). For phenobarbitone, one of the substituents is a phenyl group *(Figure 1)* (20). The substitution with the phenyl group gives it maximum anti-seizure activity (20).

Mechanism of action

Phenobarbitone is a general CNS depressant (20). It has numerous mechanisms of action, but it acts mainly through the inhibitory neurotransmitter GABA, and increases the inhibition at the synapse (20). Normally, GABA binds to the $GABA_A$ receptor chloride channel complex, which is a post synaptic receptor (20). Activation of the $GABA_A$ receptor causes opening of the chloride channel, resulting in hyperpolarization of the post-synaptic membrane (20). This reduces the continuous firing of the neurons seen in a seizure.

$GABA_A$ receptor is a ligand gated ion channel complex and is made up of 2 α, 2 β and 1 ɣ subunits (23). Barbiturates act by binding to an allosteric site on the $GABA_A$ receptor called the "barbiturate binding site" which is different from the binding site of GABA (23).

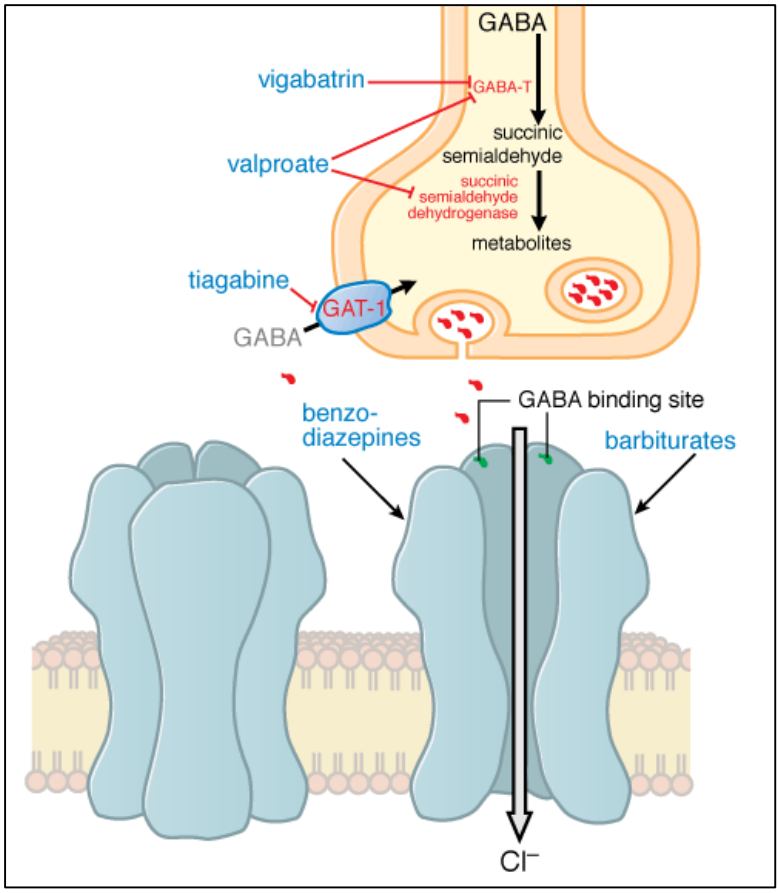
Following are the numerous mechanisms involved in the action of phenobarbitone:

1. It increases the binding of GABA to the $GABA_A$ receptor (20).
2. It increases the action of GABA on $GABA_A$ receptor. This increases the duration that the chloride channels remain open, but does not increase the frequency of opening of the channels (20).
3. At higher concentrations, it causes direct activation of the $GABA_A$ receptor and does not require GABA for its action(20) .

   Thus, phenobarbitone has both GABA-facilitatory and GABA-mimetic actions.
4. It blocks the AMPA subtype of glutamate receptors and decreases the depolarization caused by glutamate (20).

5. It blocks the P/Q type of calcium channels located pre-synaptically and decreases the release of excitatory neurotransmitters like glutamate (23).

Figure 2: Mechanism of action of phenobarbitone



Source: Brunton LL, Chabner BA, Knolmann BC: Goodman and Gilman's The Pharmacological Basis of Therapeutics, 12th edition, www.accessmedicine.com

Efficacy of phenobarbitone as an antiepileptic drug

Systemic reviews and meta-analysis comparing the efficacy of phenobarbitone to phenytoin and carbamazepine have shown that phenobarbitone is as efficacious as either of the drugs in the treatment of both the first episode of seizures as well as in epilepsy which is established (24). Currently, the WHO recommends phenobarbitone as the first line drug in both adults and children with epilepsy in the developing countries (24). This is because of the low cost

and good efficacy of the drug (24). It is included in the WHO's essential drug list as well as in the national essential drug list of majority of the responding countries (24).

Inspite of being in use as an antiepileptic drug for over a century, not many randomised control trials have been done to evaluate the efficacy of the drug (24). One of the main reasons was this is the low cost of the drug which has diminished the interest taken by the pharmaceutical companies in funding any further research (24). There are no clinical trials comparing phenobarbitone with valproate or any of the newer antiepileptic drugs (24). However, several observational studies conducted mostly in the developing countries have provided adequate evidence for its efficacy (24).

Uses

Phenobarbitone has a broad spectrum efficacy. It is used in the treatment of partial and various types of generalised seizures except absence seizures (25) . It is efficacious in the treatment of status epilepticus, idiopathic generalised epilepsy which is refractory to treatment and intractable secondarily generalised epilepsy syndromes (24). It is used as first line therapy in status epilepticus resistant to benzodiazepines and is the drug of choice in neonatal seizures (24).

Adverse effects

Neurotoxicity caused by phenobarbitone is an important concern. Sedation and drowsiness are the most common side effects reported by patients on phenobarbitone (24). However, usually patients become tolerant to the effect after a period of time (26).

Other adverse effects affecting the nervous system include ataxia, nystagmus, hypotonia, diplopia, and dizziness (13,24). An adverse effect of concern seen in children is the

paradoxical excitation with irritability and hyperactivity (24). In the elderly, it can produce agitation and confusion (20).

Impairment of cognition and depressed mood and affect are the other major concerns with phenobarbitone (27). The more uncommon side effects include megaloblastic anaemia, osteomalacia, hypersensitivity, exfoliative dermatitis and agranulocytosis (24,27).

Adverse effects like sedation are seen when the serum concentration of phenobarbitone gradually rises from 30 mg/l to 50 mg/l (28). Concentrations above 60 mg/l can result in severe toxicity (20). Children can show severe behavioural abnormalities in the absence of other signs of severe toxicity. Hence, children should not be maintained on very high doses of phenobarbitone. Only if the seizures are not controlled and the drug is well tolerated, should the concentration of phenobarbitone be increased above 30-40 mg/l (20)

Pharmacokinetics

When given orally, phenobarbitone gets absorbed almost completely (bioavailability of 95-100%). However, the absorption is slow and peak levels are attained in serum several hours after a dose (20). Its plasma protein binding is between 40-60% (20).

While upto 25% of the drug gets eliminated renally in the unchanged form, the remaining drug is metabolised in the liver, partly by oxidation and partly by N-glucosidation (26) . Oxidation takes place with the help of the Cytochrome P450 (CYP 450) enzymes (20). Phenobarbitone gets metabolised mainly by the CYP2C9, and to a lesser extent by the CYP2C19 and CYP2E1 isoenzymes (20). During metabolism, the radicals located at C5 will get oxidised to terminate the activity of the drug (20). Metabolism by the hepatic enzymes gives rise to alcohols, acids and ketones, which are eliminated in urine as glucuronide conjugates (29). The rate of metabolism of phenobarbitone in the liver is generally slow (29).

The concentration of phenobarbitone in the brain correlates well with the unbound serum concentration (27). Phenobarbitone exhibits linear kinetics i.e. the serum concentration increases linearly with the dose (26).

It has a long elimination half-life of 4-5 days (29), which enables it to be dosed once daily (27). Being a weak acid with a pK_a of 7.4, its elimination in urine can be enhanced by alkalinizing the urine (29). Alkalinisation increases the ionization of the drug in urine and decreases its reabsorption from the ultra-filtrate(29).

Both oral and parenteral formulations of the drug are available.

Pharmacokinetic variability

Phenobarbitone exhibits considerable inter-patient variability with regard to different pharmacokinetic parameters.

Absorption

When given orally in adults, phenobarbitone gets absorbed relatively rapidly (mean T_{max} = 0.5 - 4 hours) , with a bioavailability of nearly 100% (30,31). On the other hand, neonates when given phenobarbitone orally, show delayed (T_{max} = 1.5 – 6 hours) and incomplete absorption (32).

Volume of distribution and protein binding

The volume of distribution of phenobarbitone in adults is 0.54 – 0.73 L/kg with a protein binding of 50 - 60% (30,31). This is similar to the volume of distribution seen in older infants and children (0.57 – 0.70 L/kg) (32,33). Neonates and younger infants show a higher volume of distribution (0.71 – 1.17 L/kg) with lower protein binding (36 - 43% in neonates) (34–37).

Elimination in the unchanged form

Normally, about 25% of the drug is eliminated via the kidneys in the unchanged form, while the remaining is metabolised in the liver (20). However, there is considerable inter-patient variability with regard to this (38,39).

Half life

The half-life of phenobarbitone varies significantly with age. The half-life decreases gradually from 114.2 ± 40.3 hours in the first 10 days of life, to 73.2 ± 24.2 hours in the 11^{th} to 30^{th} postnatal day, to 41.2 ± 13.9 hours in the 31^{st} to 70^{th} postnatal day (40). The half-life during childhood remains fairly stable at 37 hours. In adults, the half-life increases to 73-139 hours (31,41).

Clearance

The clearance of phenobarbitone is lower in adults - 2.1 – 4.9 ml/kg/hr, when compared to children - 5.3 – 14.1 ml/kg/hr (30,31,42).

Drug interactions

A drug interaction occurs when two or more drugs are administered simultaneously. A combination of multiple antiepileptic drugs are often used in patients not controlled with monotherapy. In such patients, there is often a high chance of clinically relevant drug interactions which can change the therapeutic outcome.

Phenobarbitone is a potent inducer of the hepatic microsomal enzymes – CYP2C, CYP3A and the uridine diphosphate-glucuronosyltransferase (UGT) enzymes (20). The metabolism of drugs which are broken down by these enzymes can be increased, when co-administered with phenobarbitone. This can decrease the plasma levels of these concomitantly administered drugs, sometimes leading to sub therapeutic levels and loss of clinical efficacy

(43). On the other hand, drugs which inhibit the metabolism of phenobarbitone can increase the levels of phenobarbitone in plasma, leading to toxicity.

Following are a few clinically relevant drug interactions between phenobarbitone and other co-administered drugs:

- When phenobarbitone is coadministered with valproate, the metabolism of valproate is increased, decreasing the valproate concentration to 76% of the original concentration (44). On the other hand, the metabolism of phenobarbitone (both oxidation and glucosidation) is inhibited by valproate, increasing the plasma concentration of phenobarbitone by 30-50% in most patients (38,45,46). This can lead to sedation and drowsiness, requiring the dose of phenobarbitone to be reduced in upto 80% of the patients (38,47).
- The interaction between phenobarbitone and phenytoin is quite complex giving rise to unpredictable changes in their plasma concentrations. Both of them are metabolised by the same set of enzymes and hence they can inhibit each other's metabolism. When phenobarbitone is given in low doses, it increases the metabolism of phenytoin. On the other hand, in higher doses it inhibits the metabolism of phenytoin, increasing the phenytoin concentration (43).
- Felbamate inhibits the metabolism of phenobarbitone and increases its plasma concentration (35).
- Antacids reduce the absorption of phenobarbitone and decreases its plasma concentration (43).
- Dextropropxyphene, stiripentol and chloramphenicol increases the plasma concentration of phenobarbitone by inhibiting its metabolism (45).
- Phenobarbitone induces the metabolism of lamotrigine, topiramate, tiagabine and oxcarbazepine and decreases their plasma concentrations (43). Oxcarbazepine, being

a weak inhibitor of CYP2C19, can increase the plasma concentration of phenobarbitone (45).

- Phenobarbitone increases the metabolism of carbamazepine, reducing the plasma concentration of carbamazepine and thereby the clinical efficacy (48,49).
- Phenobarbitone increases the metabolism of oral contraceptives, anti-coagulants like warfarin and dicoumarol, corticosteroids, theophylline, antiviral drugs, cyclosporine and anticancer agents. Thus, the concentrations of these drugs decrease leading to loss of clinical efficacy (20,43).

CYP450 polymorphism

Genetic polymorphisms exist in the cytochrome P450 enzymes (50). Such polymorphisms of the CYP enzymes affect the expression of these enzymes, and thereby determine the predisposition of an individual to drug toxicity (50).

5% of Caucasians and 20% of Asians were found to be poor CYP2C19 metabolizers (51). A study conducted in Japanese patients with epilepsy showed that polymorphisms of CYP2C19 decreased the clearance of phenobarbitone (52). Another study showed that there was no difference in the metabolism of phenobarbitone between poor metabolizers and extensive metabolizers (53).

On the other hand, ~ 35% of the Caucasians and less than 10% of the Asians and the African-Americans had genetic polymorphisms of CYP2C9 (54). Even though phenobarbitone is mainly metabolized by CYP2C9 enzymes, it has not yet been reported whether CYP2C9 polymorphisms interfere with its metabolism (27).

Therapeutic drug monitoring (TDM)

Therapeutic drug monitoring is a tool for optimising drug therapy based on the relationship between the concentration of the drug in body fluids (usually serum or plasma) and the efficacy and/or toxicity (55,56). Thus it helps to optimise therapy by individualising the dose of the drug. Numerous biological fluids can be used for TDM such as serum, plasma, saliva, CSF, urine, tears or whole blood (57).

Therapeutic drug monitoring of antiepileptic's dates back to the late 1960's, when the serum or plasma concentrations of the older antiepileptic's – phenytoin and phenobarbitone were measured (57). Nowadays, the spectrum of antiepileptic drugs being monitored with TDM is wide and includes drugs like carbamazepine, valproate, ethosuximide and lamotrigine (57). TDM of antiepileptic drugs is typically done using serum or plasma (57). The concentrations are usually measured at trough steady state (57).

Criteria for a drug to be suitable for therapeutic drug monitoring (56,58)

TDM will be particularly valuable in a drug which has the following properties:

- Large inter-individual variation in pharmacokinetics (the pharmacokinetic variability can be due to genetic polymorphisms, age, gender and disease states or because of the variation in absorption, protein binding, metabolism, distribution and clearance).
- Narrow therapeutic range.
- The concentration of the drug in serum/ plasma should correlate well with the efficacy and/or toxicity of the drug.
- Poor correlation between the dosage of the drug and the serum/plasma concentration.
- The concentration of the unbound drug in serum/ plasma should correlate well or should be equal to the concentration of the drug in the brain (for antiepileptic drugs).

- Intra-individual variability in pharmacokinetics caused due to drug interactions.

When is therapeutic drug monitoring useful? (25,26,56)

- In suspected toxicity.
- In suspected sub therapeutic concentrations (patients who come with failure of therapy).
- To aid in dose adjustments in situations where there is increased pharmacokinetic variability (disease, children, elderly, pregnancy).
- To determine patient compliance.
- When a drug formulation is changed.
- To determine the effect of co-medications on drug concentration.
- To establish the concentration at which there is a good therapeutic effect. This concentration can be used at a later stage to determine the possible causes for a change in the effect of the drug.
- Following a dose adjustment especially in drugs which exhibit non-linear pharmacokinetics.

Why is therapeutic drug monitoring required for phenobarbitone?

For a patient with epilepsy, adequate control of seizures as well of prevention of toxicity due to antiepileptics is essential to ensure a good quality of life.

It is well accepted that phenobarbitone requires therapeutic drug monitoring (26,58). This is because the serum concentrations exhibit considerable inter-patient variability under the influence of age and drug interactions (26,43,45).

At present, therapeutic drug monitoring of phenobarbitone is achieved by measuring the serum or plasma concentrations (26,57). The accepted therapeutic range for phenobarbitone

in serum/plasma is 15 - 40 mg/l (57,59). Its long half-life gives it the advantage of minimum variability in its concentration during a dosing interval, provided steady state has been attained (26). Thus blood samples for TDM of phenobarbitone can be collected at any time point during the day (26).

Criteria for assays in TDM

The assay should be:

- Sensitive and specific with good precision and accuracy
- Reliable and quick
- Cost-effective

Analytical methods

Due to the development of numerous analytical techniques, the monitoring of drugs in biological matrices has been made easier. The TDM of phenobarbitone began with the use of UV spectrophotometry (57). However, the technique had the disadvantages of being cumbersome with low sensitivity and specificity (57). This was replaced by chromatographic techniques and immunoassay methods (60).

The main analytical method currently used for estimation of phenobarbitone concentration in serum is immunoassays (26). The different immunoassay methods include enzyme-multiplied immunoassay technique (EMIA), fluorescence polarisation immunoassay (FPIA), cloned enzyme donor immunoassay (CEDIA), chemiluminescent microparticle immunoassay (CMIA) and particle enhanced turbidimetric inhibition immunoassay (PETINIA) (61). Immunoassays are widely used because of the ease of use and speed (61). They have the advantage of requiring low technical ability as there is no need for manual extraction of the

drug prior to analysis (61). They are highly sensitive, but have the disadvantage of cross-reactivity of the antibodies used in the assay with the metabolites of the drug (57).

The two types of chromatographic techniques are gas liquid and high performance liquid chromatography (60). Even though gas liquid chromatography (GC) has enhanced sensitivity and specificity (57), it has the disadvantages of being highly complex in nature and being unable to adapt to a change according to the needs or the environment (60). Hence GC has largely been replaced by HPLC (62).

Currently, HPLC is the most widely used chromatographic technique for therapeutic drug monitoring (62). HPLC techniques have the following advantages (57,60):

- Sensitive
- Specific
- Ability to quantify multiple drugs in one separation
- Sometimes, the known metabolites of the drugs can also be quantified
- It is the most appropriate method to study the pharmacokinetics of a drug in order to evaluate the need for therapeutic drug monitoring

HPLC requires high degree of technical precision. The cost of analysis can be overcome if a large number of samples are analysed at the same time.

High performance liquid chromatography (HPLC) (63–67)

Sir Mikhail S. Tswett, a Russian botanist coined the term “Chromatography” in the year 1903. Chromatography is a technique used in analytical chemistry to separate the components of a mixture. Tswett found that when a plant extract carried with the help of petroleum ether, is made to flow under the influence of gravity through a vertical glass tube filled with calcium carbonate, multiple plant pigments are separated. These were observed as coloured

bands along the column. The term “Chromatography” was coined from the two Greek words “chroma”, which means “colour” and “graphos”, which means “to draw”.

The traditional (low pressure) liquid chromatography made use of pumps which generated pressures of upto 500 psi.

High pressure liquid chromatography, currently known as High performance liquid chromatography, is a form of liquid chromatography used to separate the components of a liquid mixture so as to purify, identify and quantify the individual components. It is different from the traditional liquid chromatography in the fact that it makes use of smaller columns and higher pump pressures which gives it high resolution separation and speed when separating the components of a mixture.

The term “High pressure liquid chromatography” was coined by the late Sir Csaba Horváth, a Hungarian chemical engineer in 1970. In the 1980’s, the name was changed to “High performance liquid chromatography”.

The HPLC Instrument

A HPLC instrument is typically composed of two to four pumps, an injector, a column, a detector and a data processing unit and display. The system may or may not have a column heater and the area for specimens prior to injection may or may not have temperature control.

The sample containing the analytes is introduced through the injector port into an isolated sample loop. The valves within the rheodyne system move to open the channels which bring the mobile phase into the loop. The mobile phase flows into the loop, picks up the sample and takes it to the column. As the sample moves through the column, different analytes move at different velocities which lead to the separation of the analytes. The velocity with which a particular analyte moves depends on the interaction of the analyte with the stationary and mobile phases. If the analyte has greater affinity for the mobile phase when compared to the

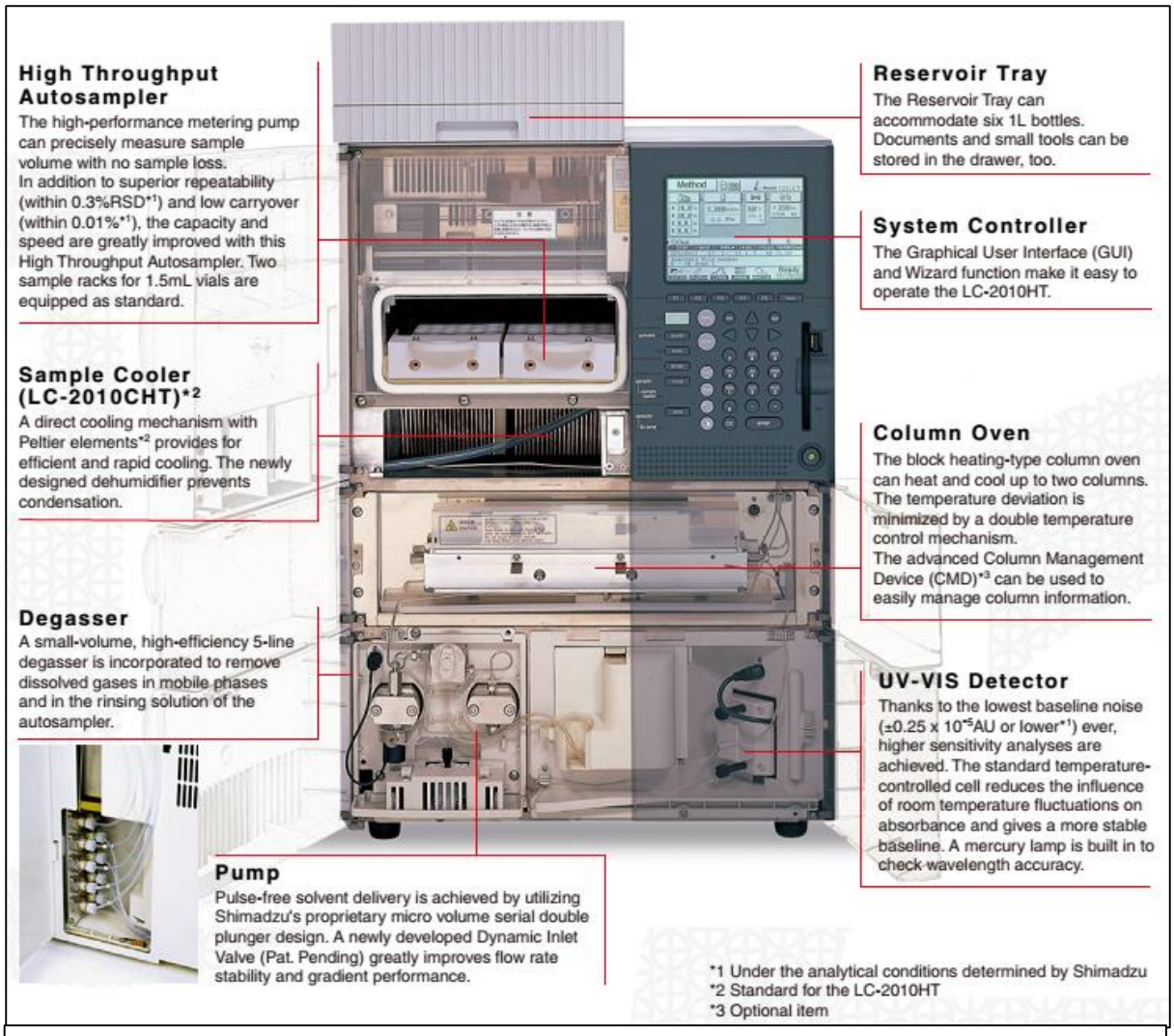
stationary phase, then it will move with greater velocity and will elute from the column earlier than analytes which have an affinity for the stationary phase.

The interaction/ affinity is based on the principle of "Like attracts Like" or polarity. If the stationary phase is polar and the mobile phase non polar, then non polar compounds will elute first, followed by the polar compounds.

The time taken for a specific analyte to emerge from the column following its injection under a particular set of conditions is fixed and is called the retention time (RT).

From the column the analytes move to the detector whose signal is represented as a chromatographic peak.

Figure 3: Automated Injector Shimadzu LC 2010 CHT- diagram of internal section



Source: Schimadzu LC 2010HT (High pressure liquid chromatograph)

Pump

The pump generates the pressure that is required to transport the mobile phase carrying the sample through the column. Current pumps can generate pressures upto 6000 psi. The pump controls the flow rate (Normal flow rate = 1-2 ml/minute for a column with an internal diameter of 4.6 mm). The pump also delivers the desired composition of mobile phase to the column. The instrument can be programmed for two different types of elution as given below.

* **Isocratic elution:**

In isocratic elution, the composition of the mobile phase is kept constant throughout the analysis. An isocratic elution is used to separate the components of a mixture which are quite similar to each other in their affinity for the stationary phase.

e.g.: 70% Water + 30% Acetonitrile throughout the time of analysis

* **Gradient elution:**

In gradient elution, the composition of the mobile phase is changed with time during the analysis. Typically in gradient elution, the composition of the mobile phase is changed from low eluting strength to high eluting strength. The high eluting strength will enable the late eluting compounds to elute out faster and have a shorter retention time.

e.g.: A gradient starting at 10% Acetonitrile in water and ending with 70% Acetonitrile in water towards the end of analysis

In isocratic elution, the peak becomes broader as the retention time increases. Thus, in this scenario the late eluting compounds will have very broad peaks, which will make it difficult to identify them as definite peaks. Gradient elution will decrease the retention time of the late eluting compounds and enable the peak shape to be better defined.

Injector

The injector will serve to introduce the sample into the moving mobile phase. Injection of the sample can be manual or automated.

An "Autosampler" or an automated injector becomes useful when there are numerous specimens to be analysed and hence the use of a manual injector becomes difficult and cumbersome. The extracted specimens are loaded onto an autosampler tray (capacity of 100 specimens). The appropriate volume of specimen is drawn into the autosampler needle, which is then transported by the mobile phase to the column. The needle is flushed prior to injecting the next sample.

Detector

Ultra-violet detector (UV detector):

It is one of the most commonly used detectors in HPLC. It is an absorbance detector.

Principle of UV detector:

At the time of analysis, the sample moves through the flow cell. A light source will emit ultraviolet light, which is irradiated through the flow cell. This ultra-violet beam falls on a sensor which is located at the other end. The sensor measures the amount of light falling on it. If an analyte eluting from the column absorbs this UV light as it flows through the flow cell, the amount of light falling on the sensor changes. The amount of light absorbed by an analyte is proportional to the concentration of the analyte.

There are two types of ultra-violet detectors – fixed wavelength and variable wavelength.

In the fixed wavelength type, a lamp is used (usually mercury vapour lamp), which emits a single wavelength. Because the intensity of the wavelength emitted is high, fixed wavelength detectors are several times more sensitive than variable wavelength detectors.

In variable wavelength detectors, a deuterium lamp is usually used, which emits a broad range of wavelength. The wavelengths are irradiated onto a diffraction grating, which functions just like a prism and separates the wavelengths. The light is now made to pass through a barrier with a tiny slit. Adjustments are made such that only a selected wavelength is allowed to pass through the slit. The selected wavelength passes through the sample and the sample absorbs part of the light.

Data processing Unit and Display

The detector provides an output, which is picked up by the data processing unit and displayed on the computer screen as a "Chromatogram". Each peak on the chromatogram denotes a separated analyte which has emerged from the column.

The Mobile and Stationary Phases in HPLC

Mobile phase

It is a liquid phase which helps to carry the specimen through the column. In reversed phase chromatography (RPLC), the mobile phase is usually a combination of water with an organic solvent like methanol or acetonitrile (polar solvents). The choice of mobile phase for an assay depends on the interaction of the mobile phase with the stationary phase and the analyte. The pH of the mobile phase can be adjusted by adding a buffer. In RPLC, the pH is usually between 2 - 8.

Buffers are important in RPLC for mainly two reasons:

1. For analytes prone to ionization, the buffer ensures that the analyte exists in a single form – ionized or unionized. This ensures a good peak shape.

2. If the pH range of the analyte is such that it can damage the column, the buffer changes it to a less harmful pH.

Phosphate buffer is the most common type of buffer. The concentration of the buffer is usually in the range of 25 to 50 mM.

Stationary phase

The stationary phase consists of an immobile solid phase packed within a column. It could be made up of either polar compounds like silica or alumni which are porous in nature or non-polar compounds like C18 and C8 which are bonded onto the surface of the porous particles. e.g.: bonded silica.

Reversed - phase Chromatography

In reversed - phase chromatography, the stationary phase is non polar (e.g.: silica bonded with –C18), while the mobile phase is polar (e.g.: methanol). Hence, the polar analytes will elute out quickly, while the non-polar analytes will be retained in the column longer, and will elute out later.

Characteristics of the column

Tubing of the column

The outer tubing of the column can be made of stainless steel, glass or PEEK polymer. Stainless steel is the most commonly used material since it enables the column to withstand very high pressures.

Chemistry of the column

The most commonly used packing material in the manufacture of stationary phase is silica, which is polar (hydrophilic) in nature. The silica can be made non-polar by bonding it with ligands like -C18, -C8, -C4 and -Cyano groups. A polar stationary phase is used in normal phase HPLC, while a non-polar stationary phase is used in reversed phase HPLC.

Carbon load - It is a measure of the amount of bonded ligands which are attached to the surface of the packing material. With a low carbon load, the retention time is usually faster.

End–capping – Silica is a polymer which terminates on the surface as – Si-OH groups (Silanols). The bonded ligands like –C 18, attach themselves to the silica through the silanols. However, because of the large size or bulkiness of the bonded ligands, all the exposed silanols are not bonded. These exposed silanols being acidic in nature, can react excessively with basic analytes and produce tailing of peaks. Hence, these unbound silanols (also called as "residual" silanols) are made less reactive by bonding it with smaller ligands. This is called as "End – capping".

Particle Size

The stationary phase is usually made up of smaller particles. As the particle size decreases, the surface area available for interaction increases, which enables better separation of the components of a mixture.

However, as the particle size decreases, the back pressure of the column increases. This necessitates the use of pumps which can generate higher pressures. The particle size in HPLC columns ranges between 2 – 25 µm. The most commonly used particle size is 5 µm.

Particle shape

The stationary phase can be made up of either uniform spherical particles or irregular particles. Spherical particles have the advantage of easy manufacturing, higher efficiency, better stability of the column and lower back pressures. The columns composed of irregularly shaped particles are usually more difficult to manufacture.

Pore size

The stationary phase is usually porous to increase the surface area available for interaction. The pore size of the particles denotes the average size of the pores which are present within each particle. As the pore size increases, the surface area available for interaction decreases. The molecular weight (size) of the analyte should be taken into consideration prior to choosing the pore size as given below:

Molecular weight < 3000 - Pore size of < 100 A°

Molecular weight of 3000 - 10,000 - Pore size of 100 – 130 A°

Molecular weight > 10,000 - Pore size of 300 A°

Column length

Longer columns provide better separation (with the same pore size). However, the run time, mobile phase consumption and back pressure increases with the length of the column. Usually, the HPLC columns are between 50 – 300 mm long.

Internal diameter of the column

Narrower columns have the advantage of better sensitivity of detection and lesser mobile phase consumption. However, only small quantities of the sample can be loaded onto the column when compared to wider columns which have a large loading capacity. Also, as the

internal diameter increases, the back pressure decreases. Hence, wider columns have the advantage of lower back pressure. Since wider columns generate lower back pressures, it enables them to have a higher flow rate. Therefore, as the internal diameter increases, the flow rate increases.

Flow rate	Column diameter
1 - 2 ml/minute	4.6 mm
0.4 – 0.6 ml/minute	3.0 mm
0.2 – 0.4 ml/minute	2.1 mm

Column Temperature

The control of column temperature becomes important in the following situations:

- High temperatures can lead to breakoff of the bonded phase from the packing material as well as dissolution of silica. Hence, the maximum temperature to which the stationary phase can be exposed should be crosschecked prior to use.
- Certain chemical compounds like enzymes and proteins may be unstable at room temperature and hence will need lower temperatures during analysis.
- The retention time for certain compounds is temperature dependent. Variation in the temperature can lead to variation in the area/ height of the peaks.

The temperature of the column can be controlled using an oven or a water bath.

Column Pressure

The column pressure is directly proportional to the viscosity of mobile phase, length of column and the flow rate. The flow rate should always be set such that it does not over-pressurise the pump. Also, a mobile phase with the lowest viscosity should be chosen.

Guard Column

The guard column is placed between the injector and the column. It filters any particulate contaminants which may be present in the sample or the mobile phase and retain it. Thus, it protects the column from contaminants which can destroy / block the column and increases the longevity of the column.

Also, the column has an inlet frit. Frits are present on either ends of the column and prevent the exposure of the packing material. In the absence of a guard column, particulate contaminants can get collected at the column inlet frit and plug the column. This can lead to tailing and splitting of the peaks, thereby affecting the analysis.

Retention time (RT)

Under a particular standardised set of conditions, the retention time is characteristic of an analyte and can be used to identify the analyte.

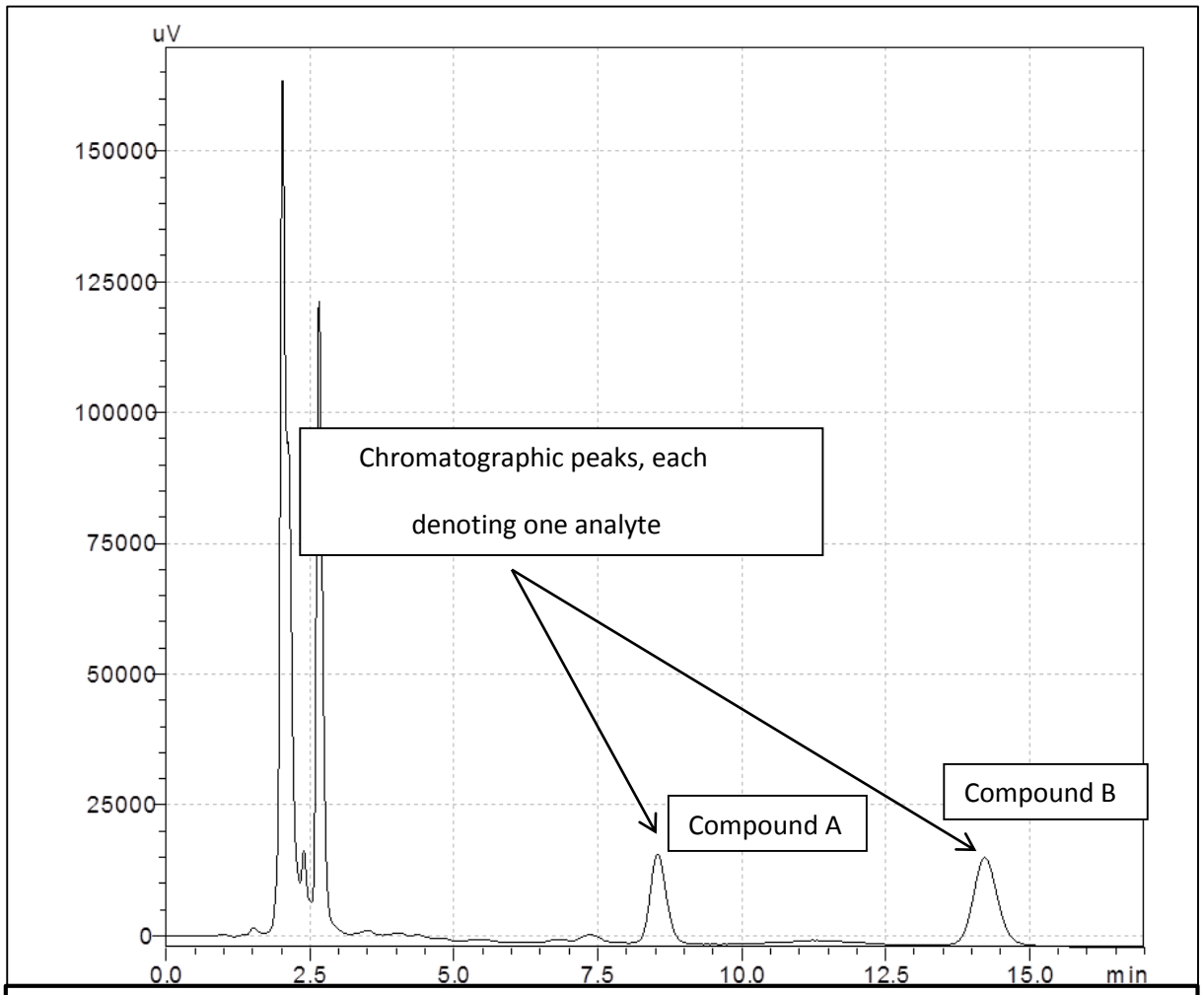
The retention time can vary with:

- Flow rate
- Composition of mobile phase
- Nature of the stationary phase
- Temperature of the column

Quantifying the analyte

The amount of analyte is quantified by measuring the area under the peak. The area under the peak is proportional to the amount of analyte. The steps in quantifying the analyte have been described in the section "Calculation of the unknown concentration of Phenobarbitone" on *pages 62 & 63*.

Figure 4: Example of 'Chromatogram'



Retention time of Compound A - 8.67 minutes

Retention time of Compound B - 14.45 minutes

Dried blood spot (DBS)

Dried blood spot refers to the analysis of whole blood spotted and dried on a filter paper (68). This technique was first described over a century ago by Ivar Christian Bang, when he used dried blood spots for measurement of blood glucose in rabbits (68). Its first clinical application came in the early 1960's, when Dr Robert Guthrie used DBS for detection of the metabolic disorder, phenylketonuria in newborns (68).

Applications of Dried blood spot

Currently, the most common application of DBS specimens is to screen neonates for genetic and metabolic disorders like cystic fibrosis, phenylketonuria, congenital adrenal hyperplasia and hypothyroidism (68). It is also used in large scale epidemiological studies to screen for various biological markers like enzymes, hormones, vitamins and specific antigens and antibodies (69). Its application in the field of therapeutic drug monitoring is a more recent development and hence not so common (69). It is also used to perform pharmacokinetic studies in children to develop a dosing regimen for drugs (70). This is a very important use of DBS analysis, since often the dosing regimen of many drugs in children is obtained from the pharmacokinetic data of adults which could lead to prescribing sub therapeutic or toxic doses (70). Also, pharmacokinetic studies in children often require repeated blood sampling with relatively large blood volumes being taken at time of each sampling (70). This is overcome by the use of DBS for analysis. In the field of clinical chemistry, numerous analytes like nucleic acids, peptides, proteins and lipids are measured using the DBS technique (68,69).

A few uses of the dried blood spot other than neonatal screening include measuring ceruloplasmin levels in Wilson's disease, HbA_1C in diabetes mellitus, CD_4 counts in HIV and thyroxin binding globulin during screening for neonatal hypothyroidism (68).

TDM of antiepileptic drugs using DBS

A number of general articles have been published on antiepileptic drug measurements from dried blood spot.

Wegner at el have shown the use of dried blood spot for measuring both lamotrigine and oxcarbazepine concentrations in pregnancy (71). Filippi et al measured the plasma concentration of topiramate in neonates by correcting the values obtained on a dried blood spot (72). The topiramate concentration in DBS was measured using liquid chromatography tandem mass spectrometry (LC-MS/MS) (72).

There were very few papers giving hard data on the correlation between the concentrations of antiepileptic's in plasma/serum and dried blood spot. Most of the papers giving this type of data are related to the newer, and not the older antiepileptic's. Soons et al found that there was a good correlation between the concentration of lamotrigine measured in plasma and DBS (73). The dried blood spot analysis was done using reversed phase HPLC (73). A study done by Marca et al in Italy found that there was no significant difference between the concentrations of phenobarbitone measured in plasma and DBS specimens (74). The above study measured the plasma concentration using immunoassay and the dried blood spot concentration using LC-MS/MS (74). Our study aimed at going a step further in predicting the phenobarbitone concentration in serum from that in DBS by formulating an equation.

Collection and Sampling

Sampling can be done using two methods:

1. ***Finger prick method*** ***(68,69,75)***

   This method involves the use of a sterile lancet to puncture the skin at the heel, toe or finger. Once the first drop of blood is discarded, subsequent drops are blotted on the premarked circles of the DBS card (filter paper). Squeezing the puncture should be avoided as it causes haemolysis and dilution of the blood with tissue fluids. The circle should be completely and homogenously filled with blood. A typical DBS card (Whatman Protein Saver #903) is made up of 5 half - inch circles, each with a capacity to hold 75 - 80 µl of blood. Only one drop of blood should be applied per circle, without touching the circle. Supersaturating, clotting or layering of the blood should be avoided. If the blood obtained with the finger prick is contaminated, haemolysed or inadequate, it cannot be used for blotting on the filter paper.

2. ***Using a micropipette*** ***(69,75)***

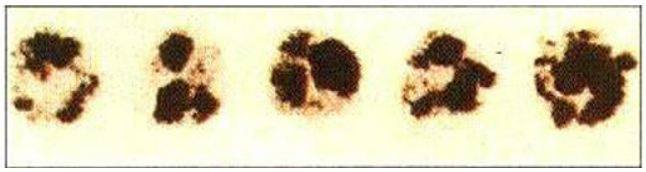
   In this method, a known volume of blood is accurately pipetted with a calibrated micropipette onto the filter paper. The tip of the pipette should not touch the filter paper and the blood should be quickly expelled as soon as the drop of blood comes in contact with the filter paper.

Drawbacks of the finger prick method

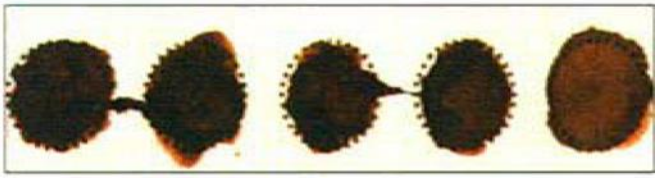
The collection of blood from a finger prick has a number of problems. A few examples are given below, all of which can lead to wastage as the specimens will have to be discarded. (76).

Figure 5: Drawbacks of the finger prick method

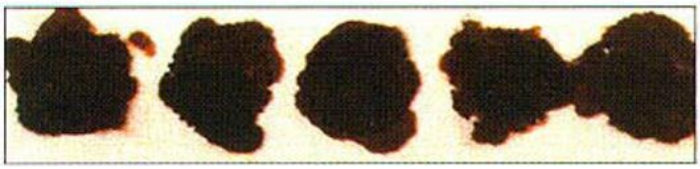
1. Inadequate volume of blood



2. Finger touching or scratching the surface



3. Clotting or layered specimens – this occurs if the blood drop is touched to the paper several times



Photos reproduced from: ACTN Laboratory Technologist Committee Version 1.1, ACTN Dried blood spots : Procedure 11 March 2009. Dried blood spots card collection, processing and storage procedures

It has been proposed that patients given clear instructions and adequate training in finger prick sampling may be able to send the DBS to the laboratory for monitoring (68). However, as Stock et al pointed out in a presentation to the International Association of Therapeutic Drug Monitoring and Clinical Toxicology, there are a number a drawbacks to this (77).

1. Sampling is not under the control of the hospital.
2. Despite training, the quality of some samples is not satisfactory and can lead to problems as shown in the figure above (Figure 5) (69).
3. Some patients have a fear of finger prick (69).

4. The small sample volume limits the number of repeat specimens.
5. The small sample volume also means that usually only one analyte can be measured per specimen.

For these reasons, we will use the second of the methods described above (using a micropipette) for spotting blood in our study.

Filter paper

A Protein Saver #903 card/paper is available from Whatman for DBS analysis (75). It is an FDA listed class 2 medical device used extensively for neonatal screening (75). However, these cards are expensive and not freely available in India. An alternative is to use the standard Whatmann filter paper.

Drying of the DBS specimen

After spotting, the DBS papers are completely air dried at room temperature on a non-absorbent surface (68). The duration for which the papers are dried depends on the type of paper and the volume of spotted blood (69). The DBS cards used for neonatal screening are air dried for a minimum of 3 hours at a temperature of 15 – 22 °C (69). During drying, the specimens should not be piled on top of each other or allowed to come in contact with any other surface (75). Exposure to direct sunlight should also be avoided (75).

It is important to completely dry the papers prior to storage, as any left-over moisture can promote bacterial growth, interfere with the elution time of the analyte or cause breakdown of unstable analytes (75,78). Drying the blood spot reduces the risk of infection by destroying the capsid of the following viruses (Hepatitis C virus, Human T-lymphotropic virus, Human immunodeficiency virus, Cytomegalovirus) (68).

Storage of the DBS specimen

After drying, the DBS specimens should be stored in zip lock bags with low gas permeability along with a desiccant to prevent the accumulation of moisture (75). During storage, either an individual zip lock bag should be used or the specimens should be separated from each other using a thick paper to reduce the risk of cross-contamination (68). A humidity indicator should also be placed in the storage package (75). Thus if properly stored, the DBS specimens will remain stable at room temperature for several weeks, months or years depending on the analyte (75).

Transport of the DBS specimen

Once the DBS specimens are completely dried, they can be transported by regular mail without any biohazard risk (69,75) . They should be packed in the zip lock bags as mentioned above with a paper overlaying the specimen, and sent by mail in a good quality bond envelope (75).

Methods for measuring the analyte

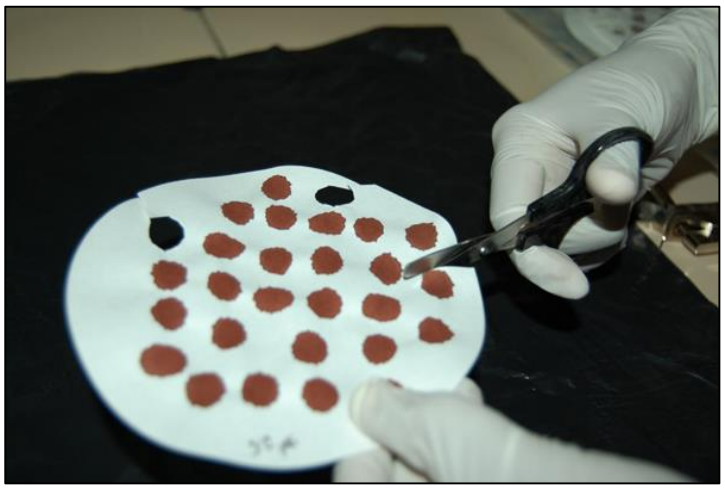
There are two methods for measuring the DBS (69).

The first method is called the "Disc punch method". This makes use of a specially designed punching device to cut out a 2-8 mm (diameter) disc from the filter paper (68,69). This disc is subjected to analysis. The disc depending on the diameter, will contain a specific volume of blood similar to that of a liquid measurement (69). This method must be used if the specimen is collected from a finger prick.

Figure 6: Disc punch method

The second method is to cut out the whole DBS from the filter paper and use it for the extraction as shown in *figure 7* (69). The second method can be used for specimens created from dropping a known volume of whole blood onto the filter paper (69). Since the volume is known, there is no need to apply any correction factor for the differing weights in the filter paper (69). This method must be used for specimens spotted using a micropipette.

Figure 7: Cutting out the whole blood spot (second method)



<u>Stability of DBS</u>

DBS specimens if stored properly, are stable at room temperature for several weeks to years depending on the analyte (75).

Studies show that the minimum stability period for majority of the analytes in a DBS is 7 days at room temperature (68). DBS has been found to increase the stability of certain compounds like opioids (68). Nucleic acids can be stored in a DBS for several months at room temperature, and for several years at – 20 °C (68). Glycated haemoglobin can be analysed from DBS upto 15 days after spotting (68). Lipids, sugars and small organic and inorganic molecules like vitamin D and triglycerides remain stable for a duration of 30 days at room temperature (68). At a lower temperature of 4 °C, they can be stored for upto 90 days (68). An FDA validated DBS method which can quantify nine anti-retroviral drugs showed the stability of these drugs for 12 to 90 days at room temperature (68).

Numerous assays developed for quantifying drugs from DBS report the stability of these drugs in the dried blood (69). Amodiaquine, the antimalarial drug was found to be stable for upto 30 days at 4 °C and 1 day at room temperature (69) . While cyclosporine was found to be stable upto 4 weeks at room temperature, dapsone was stable for 3 months when stored between – 20 °C to + 35 °C (69).

Advantages of DBS for analysis (68,69,75,79)

The advantages of DBS include:

i. A smaller volume of blood is required for DBS analysis when compared to using the conventional serum/plasma.
ii. It is a relatively non-invasive method if the finger or heel prick is used for blood collection.
iii. It reduces the risk of infection (biohazard risk) during handling to a minimum.
iv. Being stable at room temperature it does not require freezers or dry ice for storage, unlike serum and plasma.

v. Because of the stability and the low risk of infection, DBS specimens can be easily transported in a cheap manner without the need for cold chain. They can be transported by ordinary mail to the laboratory for analysis.

vi. The whole blood sample collected for DBS does not need to be centrifuged, separated or frozen immediately (unlike the sample collected for serum/plasma analysis).

vii. If adequately trained, patients can themselves carry out the sampling at home.

Disadvantages of dried blood spot for analysis (68,69)

The disadvantages of DBS include:

i) Since very small blood volumes are used for analysis, the sensitivity of the assay should be high.

ii) The small sample volume limits the number of repeat specimens.

iii) Despite adequate training, the sampling when done by the patient often does not meet the required standard leading to wastage of specimens.

iv) If very low concentrations of analytes need to be measured, extremely sensitive analytical techniques like mass spectrometry will be required. This increases the cost of analysis

v) The haemoglobin and other intracellular matter released from haemolysed RBC's can produce interferences in the assay.

vi) The proteins in the blood can get denatured during drying thereby changing the enzymatic activity of the proteins.

vii) The analysis of DBS specimens gives the concentration of the analyte in whole blood. However, the therapeutic range for most drugs has been reported in serum/plasma. Hence a therapeutic range of the drug in whole blood should be

defined or conversion of the whole blood values to serum/plasma values should be done by means of an equation.

viii) Haematocrit has been found to influence the dispersion of the blood in the filter paper. Hence, haematocrit needs to be taken into account when calculating the concentration.

Summary and justification for the study

Phenobarbitone is a commonly used antiepileptic for the treatment of partial and various types of generalised seizures. It is well accepted that phenobarbitone requires therapeutic drug monitoring due to its variable pharmacokinetics and numerous clinically relevant drug interactions.

Phenobarbitone is usually measured using serum or plasma levels. An alternative method to measure drug concentrations, which is gaining popularity in the therapeutic circles, is the use of DBS. The advantage of dried blood spot over serum is that these samples are stable, easy to store and transport. They are less of a biohazard risk to handlers when compared to whole blood, plasma or serum.

The aim of this study is to determine if an equation needs to be developed that will accurately predict the concentration of phenobarbitone in serum from that measure in dried blood spot. The reason for this is that the therapeutic range is for serum and not for dried blood spot.

Patients who are on phenobarbitone and who fulfil the inclusion criteria will be enrolled into the study after obtaining informed consent. Blood is collected by venepuncture for analysis. Whole blood (20 µl) will spotted on the standard Whatman filter paper, which is then air dried and the concentration measured by High Performance Liquid Chromatography. Simultaneously, the concentration of phenobarbitone in serum is measured by HPLC. If the

concentration of phenobarbitone can be accurately predicted from a dried blood spot specimen, then this can be used by smaller or rural hospitals to send samples for phenobarbitone monitoring to a higher center, in a safer and cheaper manner.

The correlation will be determined between the phenobarbitone concentration in the DBS specimen and that in serum for all the patients. The patients will be divided into two groups of approximately 50% by random allocation. The first group will be used to generate a regression equation and the second group will be act a validation group. Validation will be performed by bootstrap method.

Methodology

Chemicals and reagents

Phenobarbital pure powder was obtained from the pharmacy of Christian Medical College and Hospital, Vellore. The internal standard used for the dried blood spot assay – 4 - aminoacetophenone was purchased from Sigma Aldrich, India. The internal standard used for the serum assay – Ornidazole, was also obtained from the pharmacy of Christian Medical College and Hospital, Vellore.

Acetonitrile (ACN) and methanol used for the assay were of HPLC grade and supplied by Thermo Fisher Scientific Private Limited, Mumbai, India. The phosphate salts used in the preparation of the buffer – di-potassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen orthophosphate (KH_2PO_4) were also of HPLC grade and obtained from Thermo Fisher Scientific Private Limited, Mumbai, India.

Deionised water purified using the Millipore Milli-Q apparatus (Merit Enterprises, Chennai, India) was used for the assay.

Equipment

- Automated Injector High Performance Liquid Chromatography LC – 2010CHT instrument with UV detection [Shimadzu Analytical (India) Pvt. Ltd.].
- HPLC Column - 4.6 mm * 250 mm C -18 column (5 µm particle size, 300 A° pore size), purchased from Thermo Fischer Scientific India Private Limited, Mumbai, India.
- HNN membrane disc filter with a pore size of 0.22 micrometre (Nupore Filtration Systems Private Limited, Ghaziabad, India) - required for the filtration of the prepared buffer and the organic solvents - Acetonitrile and Methanol. The HNN membrane disc filters are biologically inert and hydrophilic in nature.

- Filtration apparatus – comprising of a membrane filter holder (membrane diameter – 47 mm) and a conical flask (volume – 1000ml) from Science house, Chennai, India.
- Qualitative Grade 1 Whatman filter paper, diameter 125mm (GE Healthcare UK Limited, United Kingdom) - used for spotting whole blood.
- Single tube vortex (Model – CM 101, Science House, Chennai, India).
- Centrifuge – Rota 4R-V/Fm (Science House, Chennai, India).
- Micropipettes (Model – Erba Biohit (1-CH), Transasia Bio-medicals Ltd., Mumbai, India) - Adjustable for volumes 20 µl - 1000 µl.
- Micropipette tips (Tarsons Products Pvt. Ltd., Kolkata, India).
- Disposable 1.5 ml eppendorf tubes (Tarsons Products Pvt. Ltd., Kolkata, India) for extraction.
- Disposable 350 µl plastic vials (Crescent Scientific Private Limited, Chennai, India).
- Ultra-sonicator (Model – 9L-250, Associated Instruments and Chemicals, Chennai, India) for degassing mobile phases.

HPLC conditions and validation of the assays

A. Serum

HPLC conditions for extraction of phenobarbitone from serum

Instrument – Automated Injector Shimadzu LC 2010 CHT

Column – Discovery C18 (5 µm, 300 A°, 250 mm * 4.6 mm)

Buffer – 29 mM phosphate buffer (pH – 6.9)

Mobile phase – 20% acetonitrile + 80% buffer

Isocratic elution

Flow rate – 1.3 ml/minute

Wavelength – 214 nm, 254 nm (UV detector)

Loop – 20 µl

Temperature – Ambient

Preparation of drug stock solution (for standards and QC preparation)

A stock solution of phenobarbitone - 1 µg/µl, was prepared by dissolving 100 mg of pure phenobarbitone powder in 20 ml of Isopropanol and make up the volume to 100 ml with water. Separate stock solutions of 1 µg/µl each were made for preparation of standards and quality control (QC).

Preparation of serum standards and quality control

From 1 µg/µl standard stock(1), the following working standards were prepared: 50, 30, 20, 10, 5 & 2.5 µg/ml

Std 50 µg/ml = 200 µl of Std stock 1 µg/µl + 3800 µl of calf serum

Std 30 µg/ml = 120 µl of Std stock 1 µg/µl + 3880 µl of calf serum

Std 20 µg/ml = 80 µl of Std stock 1 µg/µl + 3920 µl of calf serum

Std 10 µg/ml = 40 µl of Std stock 1 µg/µl + 3960 µl of calf serum

Std 5 µg/ml = 20 µl of Std stock 1 µg/µl + 3980 µl of calf serum

Std 2.5 µg/ml = 1000 µl of 5 µg/ml + 1000 µl of calf serum

A separate stock was made for the Quality Control specimens.

From 1 µg/µl QC stock, working QC – 15 µg/ml was prepared:

Qc 15 µg/ml = 60 µl of QC stock 1 µg/µl + 3940 µl of calf serum

From 1 µg/µl QC stock, the following concentrations were prepared to be used in the validation process as low, medium and high QC:

Low QC (7 µg/ml) = 35 µl of Std stock 1 µg/µl + 4965 µl of calf serum

Medium QC (20 µg/ml) = 80 µl of Std stock 1 µg/µl + 3920 µl of calf serum

High QC (50 µg/ml) = 200 µl of Std stock 1 µg/µl + 3800 µl of calf serum

Preparation of internal standard for extraction from serum

A stock solution of internal standard (IS) – Ornidazole (1µg/µl) was prepared as follows:

2 mg of Ornidazole pure powder dissolved in 2 ml of acetonitrile = 1 µg/µl of Ornidazole stock.

Preparation of extraction solvent containing internal standard for extraction from serum

30 µl of 1 µg/µl of Ornidazole stock + 970 µl of acetonitrile = 30 µg of Ornidazole in 1 ml of ACN

Extraction

The specimens were extracted by the precipitation method. There were certain considerations taken into account.

1. Which solvent/solution should be used for precipitation? For example acetonitrile, methanol or trichloroacetic acid is commonly used.
2. Our analyte (drug of interest) must be soluble and stable in the precipitating solvent chosen.
3. What is my biological matrix and does precipitation remove interfering substances present in the biological matrix adequately (assessed by running blank serum specimens in the HPLC conditions)?

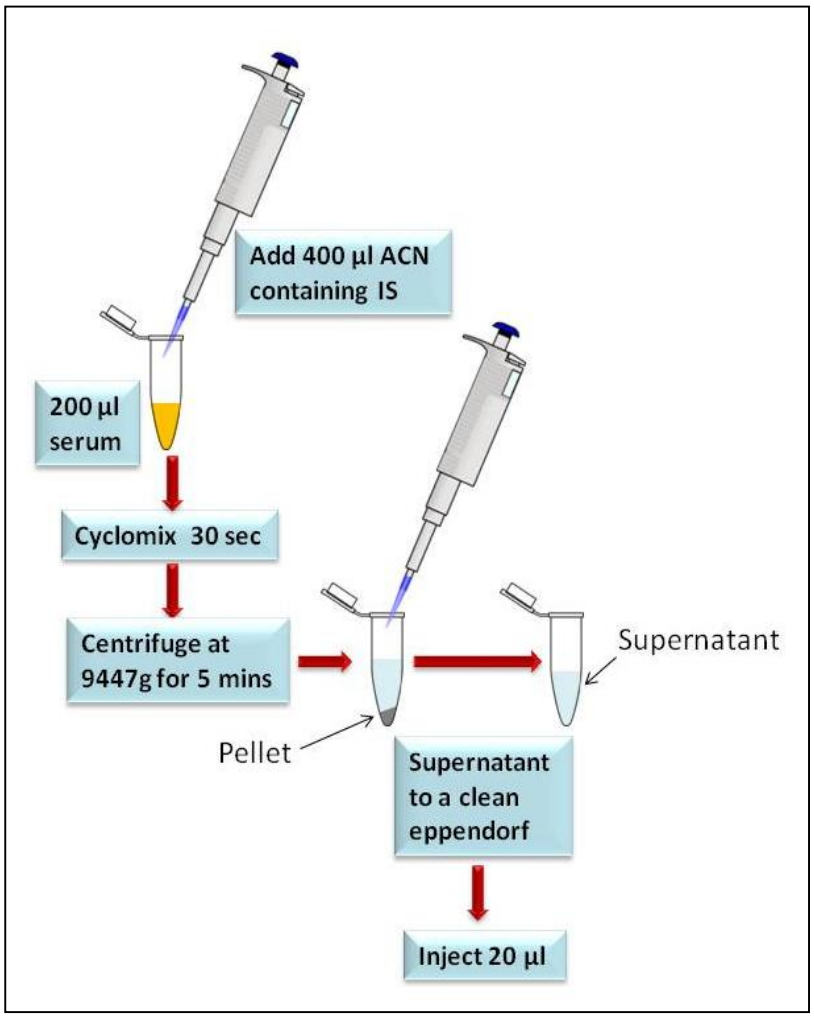
4. Does precipitation alone give the sensitivity required for the analyte or is concentration to dryness and reconstitution required?

Extraction by precipitation

Extraction by precipitation is one method used to extract drugs from biological matrices. Following the addition of solvent to the biological matrix containing the analyte, the mixture is vortexed (cyclomixed) at high speed for 1 minute. During this time precipitation of the proteins occurs. The specimen is then centrifuged at 9447g for 5 minutes to reduce the protein precipitate and other matrix components to a hard pellet thus reduces the interfering compounds in the analysis. The clear supernatant containing drug may be removed easily from this pellet and placed in a clean eppendorf. To concentrate the analyte this supernatant is evaporated to dryness. It is then reconstituted in mobile phase or an appropriate solvent, and injected.

The steps in the extraction of phenobarbitone from serum are shown in *figure 8.*

Figure 8: Serum Assay for Phenobarbitone



Development and validation of the assay for the extraction of phenobarbitone from serum

Validation of analytical methods is performed to ensure that the methodology used is reliable and reproducible when used to quantitatively measure the analyte in the biological matrix of interest.

A) Water standards – To determine the retention time of the analyte and Internal standard

Water standards of the drug and the internal standard are prepared by spiking deionised water (Millipore water) with the analyte (drug) and the internal standard respectively. Using these

standards the retention time of the drug and the internal standard under a particular set of HPLC conditions are determined. Water standards containing the drug – phenobarbitone and the internal standard – Ornidazole were prepared and analysed to detect the retention time.

B) Blank matrix specimens

Blank specimens are matrix specimens without the drug or the IS. They are analysed to ensure selectivity of the analyte i.e. we should be able to differentiate and quantify the analyte even when other components are present in the sample.

Blank specimens of serum were obtained from 10 different sources. The blank specimens were obtained both from a similar cohort of patients (patients on antiepileptics other than phenobarbitone) as well as from patients who were not on anti-epileptics, but on other drugs.

These blank specimens were analysed for the presence of interference under the drug and the IS peak in the chromatogram. The chromatogram traces from the blank specimens were compared against that for std 5. From this interference from the following antiepileptics was excluded – phenytoin, carbamazepine, valproate, oxcarbazepine, lamotrigine, levetiracetam, clonazepam, clobazam and topiramate.

C) Non-zero specimens – expected concentration range

Specimens from 10 different patients on phenobarbitone were analysed to determine an expected range in the concentrations of the study specimens.

D) Calibration curve - Linearity

Calibrators (standards) were prepared by spiking serum with known concentrations of phenobarbitone. Based on the analysis of non-zero specimens, the expected range of concentrations in the study specimens were found to be between 2.5µg/ml to 50µg/ml.

Six calibrators were prepared – 50, 30, 20, 10, 5 & 2.5 µg/ml from the standard stock solution. The QC - 15µg/ml was prepared from a separate QC stock solution. The preparation of the calibrators and QC is shown in the section – (Preparation of serum standards and quality control, *page 56 & 57*).

6 different calibration runs were performed over a span of several days using freshly prepared standards to ensure linearity of the standard curve.

E) <u>Intra-day variability (same batch, same day)</u>

On the same day, the following analysis was performed:

Standard 5: Extracted 5 times from a single aliquot and the extraction analysed.

Standard 50: Extracted 5 times from a single aliquot and the extraction analysed.

The % coefficient of variation (%CV) was calculated at each concentration. This test checks the intra – day variation in the assay at the high and low concentrations.

F) <u>Inter-day variability (same batch, different days)</u>

The inter-day variability in the assay was assessed by using low (7 µg/ml), medium (20 µg/ml) high (50 µg/ml) concentration spiked specimens.

The preparation of these specimens is shown in the section – (Preparation of serum standards and quality control, *page 56 & 57*).

High Concentration: Day 1: Three extractions performed and run by HPLC.

Day 2: Three extractions performed and run by HPLC.

The medium and low concentrations were treated in the same way. The % CV was calculated for each concentration.

G) Reproducibility of injection

Reproducibility of injection was checked by using a high concentration and a low concentration.

Standard 50 was extracted once and 5 repeat injections were given from the same extract.

Standard 5 was extracted once and 5 repeat injections were given from the same extract.

% CV was calculated for each concentration. The repeat injections from the same extract checked the reproducibility of the instrument.

H) Sensitivity

Sensitivity refers to the lowest concentration of analyte which can be measured within the acceptable limits of precision and accuracy (80). As this is an assay for drug monitoring and the therapeutic range is 15 – 40 µg/ml, any concentration ≤ 10µg/ml is very low and it is therefore not necessary to calculate a lower limit of quantification (LLOQ). The lowest standard quantitated in the serum was 2.5 µg/ml.

I) Stability

Phenobarbitone is known to be stable in serum/ plasma for a period of four weeks when stored at - 20 °C (81,82)

Calculation of the unknown concentration of phenobarbitone

The ratio of the area of the drug peak with that of the internal standard, is calculated for all the standards and QC. A linear regression line is plotted with the concentrations on the x – axis and the ratios on the y-axis. The slope, intercept and correlation coefficient is calculated from the regression line.

The unknown concentration of phenobarbitone in serum (and dried blood spot) is calculated using the linear regression equation y= bx + a where :

a = calculated intercept based on your standard curve

b = calculated slope based on your standard curve

The unknown concentration is calculated by: [Unknown Concentration Ratio – a] / b

HPLC conditions and validation of the assays

B. Dried blood spot

HPLC conditions for extraction of phenobarbitone from dried blood spot

Instrument – Automated Schimadzu LC 2010 CHT

Column – Thermo Scientific C18 (5 µm, 300 A°, 250 mm * 4.6 mm)

Buffer – 29 mM phosphate buffer (pH – 6.9)

Mobile phase – 19% acetonitrile + 81% buffer

Elution: Isocratic

Flow rate – 1.2 ml/minute

Wavelength – 214 nm, 254 nm (UV detector)

Loop – 20 µl

Temperature – Ambient

Preparation of Dried blood spot standards and quality control

From the 1 µg/µl standard stock, the following working standards were prepared – 40, 30, 15, 5 & 2.5 µg/ml.

Std 40 µg/ml = 80 µl of Std stock 1 µg/µl + 1920 µl of whole blood

Std 30 µg/ml = 60 µl of Std stock 1 µg/µl + 1940 µl of whole blood

Std 15 µg/ml = 30 µl of Std stock 1 µg/µl + 1970 µl of whole blood

Std 5 µg/ml = 20 µl of Std stock 1 µg/µl + 3980 µl of whole blood

Std 2.5 µg/ml = 1000 µl of Std 5 µg/ml + 1000 µl of whole blood

From the 1 µg/µl quality control stock, the working QC – 10µg/ml was prepared:

QC (10 µg/ml) = 20 µl of QC stock 1 µg/µl + 1980 µl of whole blood

From 1 µg/µl QC stock, the following concentrations were prepared to be used in the validation process as low, medium and high QC:

Low QC (7 µg/ml) = 21 µl of Std stock 1 µg/µl + 2979 µl of whole blood

Medium QC (20 µg/ml) = 20 µl of Std stock 1 µg/µl + 980 µl of whole blood

High QC (35 µg/ml) = 35 µl of Std stock 1 µg/µl + 965 µl of calf whole blood

Preparation of dried blood spot

For whole blood standards, 20 µl of whole blood was spotted immediately after preparing the whole blood standards. It was spotted onto a qualitative grade 1 Whatman filter paper using a calibrated micropipette. The blood spot was air dried at room temperature for a minimum of 3 hours or left overnight. While spotting and drying of the blood spot, care should be taken to make sure that the spotted blood does not come in contact with any surface.

For spotting patient specimens, the whole blood was spotted immediately after taking the blood specimen, and left to dry as above.

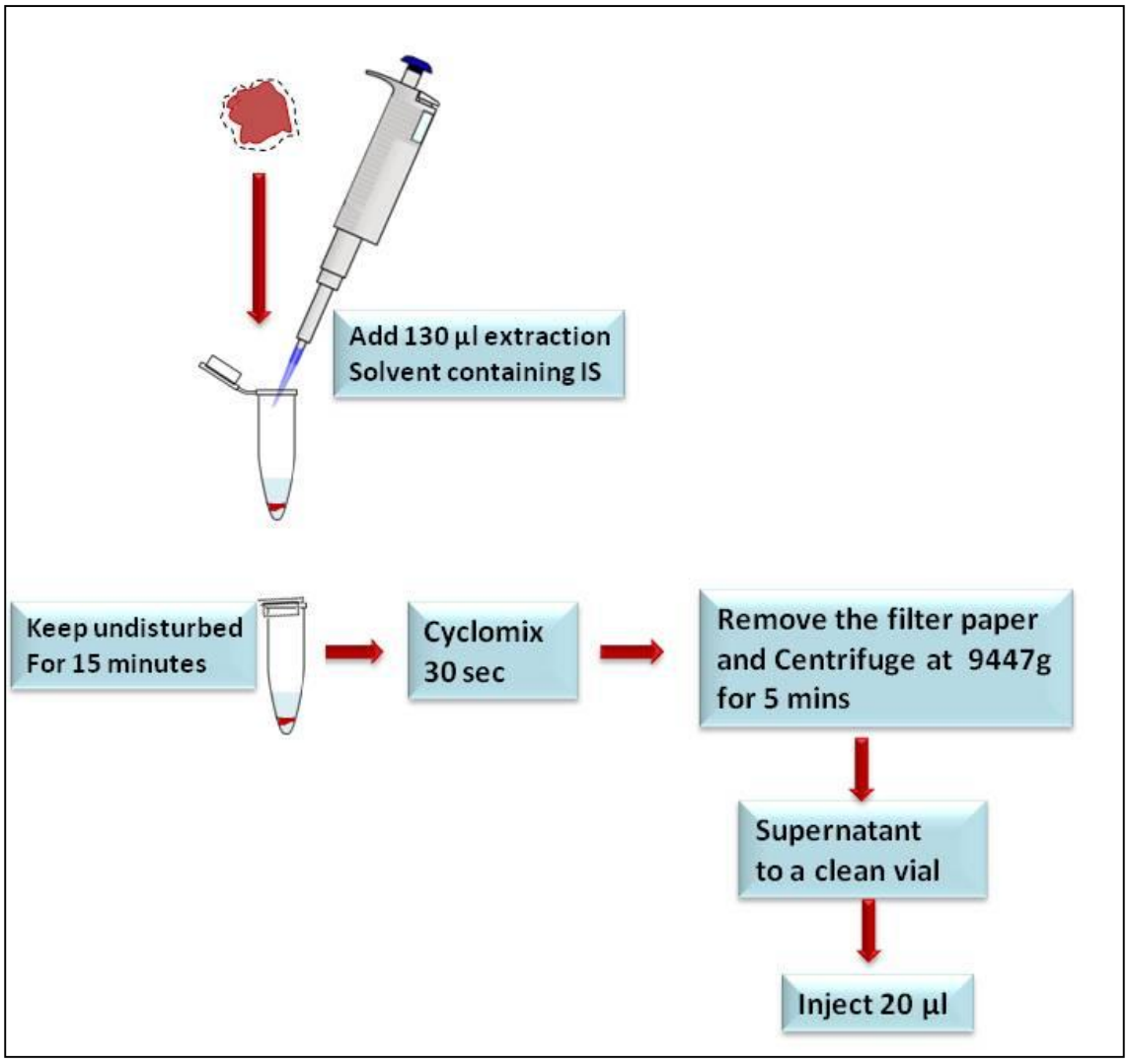
Preparation of Internal standard for extraction from dried blood spot

A stock solution of internal standard (IS) – Aminoacetophenone (1 µg/µl) was prepared by dissolving 2 mg of Aminoacetophenone pure powder in 2 ml of acetonitrile..

Preparation of extraction solvent containing internal standard for extraction from dried blood spot

Acetonitrile and Methanol (50%: 50%) + 10 µl of 1 µg/µl of Aminoacetophenone stock to make up 1 ml of the extraction mixture.

Figure 9: Extraction of phenobarbitone from dried blood spot



Correction of DBS phenobarbitone concentration for haematocrit value

The percentage of plasma was calculated for both the standards and patient specimens as follows:

% Plasma = 100 – Haematocrit

The corrected DBS phenobarbitone concentration was calculated using the formula:

Corrected DBS concentration = Final DBS concentration * (Standard plasma % / Patient plasma %)

Development and validation of the assay for the extraction of phenobarbitone from dried blood spot

A) Water standards – To determine the retention time of the analyte and Internal standard

Water standards containing the drug – phenobarbitone and the internal standard – Aminoacetophenone were prepared and analysed to detect the retention time of the drug and the IS.

B) Blank matrix specimens

Blank specimens of dried blood spot were obtained from 10 different sources. The blank specimens were obtained both from patients on antiepileptics other than phenobarbitone as well as from patients who were not on antiepileptics, but on other drugs.

These blank specimens were analysed for the presence of interference under the drug and the IS. The blank specimens were compared against the lowest standard – Std 2.5. These specimens were analysed to exclude interference due to the following antiepileptics – phenytoin, carbamazepine, valproate, oxcarbazepine, lamotrigine, levetiracetam, clonazepam, clobazam and topiramate.

C) Non-zero dried blood spot specimens – expected concentration range

Dried blood spot specimens from 10 different patients on phenobarbitone were prepared and analysed to determine an expected range in the concentrations of the study specimens. Based

on the analysis of these specimens the expected range of concentrations was found to be between 2.5 µg/ml to 40 µg/ml.

D) Calibration curve - Linearity

Calibrators (standards) were prepared by spiking whole blood with known concentrations of phenobarbitone. Based on the analysis of non-zero specimens, the expected range of concentrations in the study specimens were found to be between 2.5 µg/ml to 40 µg/ml.

Five calibrators were prepared – 40, 30,15,5 & 2.5 µg/ml from the standard stock solution. The QC - 10µg/ml was prepared from a separate QC stock solution. The preparation of the calibrators and QC is shown in the section – (Preparation of dried blood spot standards and quality control, *page 63 & 64*).

8 different calibration runs were performed over a span of several days using freshly prepared standards to ensure linearity of the standard curve.

E) Recovery of analyte from the Dried Blood spot – Extraction Efficiency

Aqueous standards and QC

Aqueous standards were prepared using the primary and secondary standard and QC stocks.

Primary standard stock – 1 µg/µl (prepared as mentioned in Section - Preparation of drug stock solution, *page 56*)

Secondary standard stock – 0.1 µg/µl = 100 µl of primary Std stock 1 µg/µl + 900 µl of water

Primary QC stock - 1 µg/µl (prepared as mentioned in Section - Preparation of drug stock solution *page 56*)

Secondary QC stock – 0.1 µg/µl = 100 µl of primary QC stock 1 µg/µl + 900 µl of water

Preparation of aqueous standards and Quality Controls (QC)

Std 40 µg/ml = 40 µl of primary Std stock + 960 µl of water

Std 30 µg/ml = 30 µl of primary Std stock + 970 µl of water

Std 15 µg/ml = 150 µl of secondary Std stock + 850 µl of water

Std 5 µg/ml = 50 µl of secondary Std stock + 950 µl of water

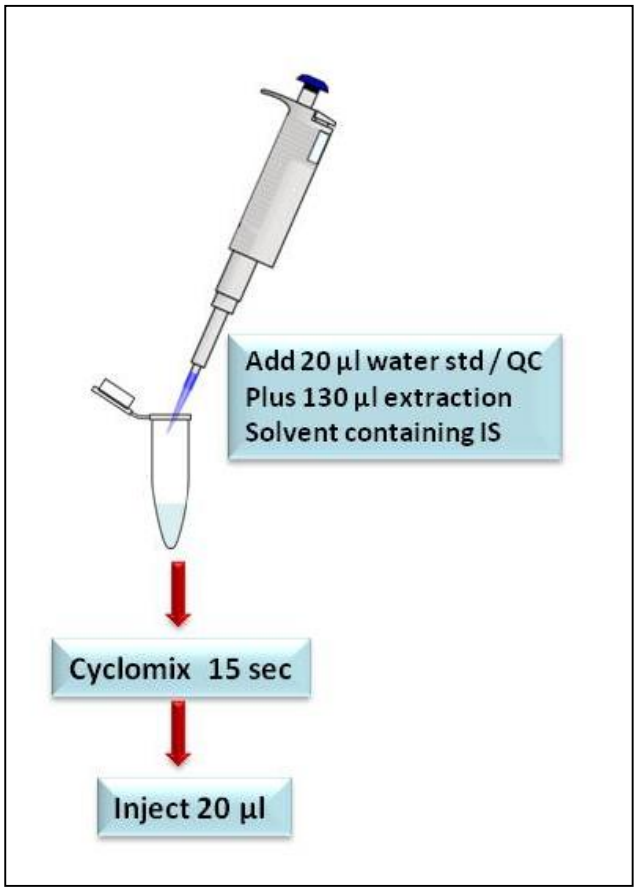
Std 2.5 µg/ml = 25 µl of secondary Std stock + 975 µl of water

Two QC's were prepared:

QC 10 µg/ml = 100 µl of secondary QC stock + 900 µl of water

QC 25 µg/ml = 25 µl of primary QC stock + 975 µl of water

Figure 10: Extraction of aqueous standards



Analysis

The aqueous standards were analysed by HPLC and the ratio of the area of drug and the IS was calculated. The dried blood spot calibrators and two dried blood QC's were also analysed. The % recovery was calculated as follows:

Step 1: Intercept and slope for the aqueous standard curve is calculated.

Step 2: Using the ratios from the DBS analysis, the concentration is calculated using the standard curve from the aqueous standards.

% Recovery = [Mean of (Concentration from DBS/ Known concentration)]* 100

F) Intra-day variability (same batch, same day)

On the same day, the following analyses were performed:

Standard 2.5 µg/ml (low concentration): Extracted 5 times from a single standard and analysed.

Standard 40 µg/ml (high concentration): Extracted 5 times from a single standard and analysed.

To check the intra-day reproducibility the % coefficient of variation (% CV) was calculated

G) Inter-day variability (same batch, different days)

This was assessed using 3 concentrations - low (7 µg/ml), medium (20 µg/ml) and high (35 µg/ml).

The preparation of the QC's is shown in the section – (Preparation of dried blood spot standards and quality control, *pages 63 & 64*).

Each concentration was extracted 3 times on day 1 and then another 3 extractions were performed on Day 3. After assay the results were compared and the % CV was calculated for each concentration. The extractions were from the same standards on Day 1 and Day 3.

H) Reproducibility of injection

Reproducibility of injection was checked using the same high, medium and low concentrations as above. Each concentration was extracted once and from this 3 repeat injections were made from the single extract.

The % CV was calculated for each concentration.

I) Sensitivity

The lowest standard of phenobarbitone that was measured from dried blood spot was 2.5 µg/ml. The chromatogram shows a clear baseline under this concentration.

J) Stability

The stability of phenobarbitone in dried blood spot were analysed at 7 days, 19 days and 26 days.

A set of standards and QC were freshly prepared on Day 1. After drying, the calibration curve was run. Linearity was good. The Quality control specimen was run and was satisfactory. These DBS specimens were then placed in plastic bags and the stability was checked for the following conditions:

Storage at laboratory temperatures (24 - 30° C).

Storage and transport at (35 - 40° C). The DBS specimens in a sealed plastic bag were driven around in a car for the period of stability testing.

New standards were freshly prepared at 7 days and 26 days. At each of these time points, the freshly prepared standards were compared against the lab standards and the car standards to assess stability. On day 19 the laboratory and car standards were compared.

Patient methodology

Institutional Review Board Clearance

The study was approved by the Institutional review board of Christian Medical College and Hospital, Vellore (IRB number – 8654).

Patient population

This was an open label, prospective study conducted in the Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore in collaboration with the Department of Neurology. Inclusion criteria were patients in the age group 18 to 65 years and receiving phenobarbitone for epilepsy. After informed consent was obtained, patients were recruited from the outpatient setting of the Department of Neurology. All patients who were prescribed phenobarbitone and who were taking it regularly were included in the study. The brand and the dose of phenobarbitone prescribed were based on the clinician's discretion and were of no consequence to the study. The compliance to medication was confirmed prior to including the patient in the study. The timing of the specimen collection could be at any time point (and not necessarily the trough concentration), provided the patient was compliant with the medication. Upon inclusion in the study, each patient was given a study number. Data from the study was stored under the patient study number. The following demographic and medication details were also noted at the time of inclusion: age, sex, concomitant medications and dose of phenobarbitone.

Calculation of sample size

The required sample size to show a relationship of phenobarbitone in serum and dried blood spot was found to be 74 subjects with 80% power and 5% level of significance with an anticipated correlation of 0.85.

Within the time allotted for recruitment and analysis of the specimens, 36 patients were enrolled in the study. This dissertation presents the results of the 36 patients. The study continues.

Collection of Blood and Serum Specimens

Figure 11: Algorithm for sample collection, separation and storage

Haematocrit estimation

At the time of sample collection from the patient, 1 ml of whole blood was immediately sent for packed cell volume estimation (PCV) to the Department of Transfusion Medicine and Immunohematology. PCV was estimated by the Unicel D*H 800 coulter cellular analysis system. The PCV was also estimated at the time of preparation of standards for dried blood spot analysis.

Preparation of Serum Specimen

2 ml of whole blood was collected into a BD vacutainer plain tube and after standing to allow clotting the specimen was centrifuged using a doctor centrifuge at step 1 (5 step regulator) for 3 minutes and the serum separated into clean eppendorf tubes.

Preparation of dried blood spots

For spotting patient specimens: 20 µl was spotted onto a qualitative grade 1 Whatman filter paper using a calibrated micropipette. The whole blood was spotted immediately after taking the blood specimen, and left to dry at room temperature for a minimum of 3 hours or left overnight.

Determination of the relevance of spotting inconsistencies

In order to evaluate the importance of the correct technique of spotting, DBS specimens which were incorrectly spotted were analysed and the concentrations obtained were compared graphically and statistically against the corresponding serum concentrations. The methods employed were, precision, bias, Spearman ranked correlation, Bland-Altman plot and Mann Whitney-U test. These are all outlined below.

Statistical analysis and graphical plots

The following statistical analyses were applied using R program language (version 3.1.5).

- Frequency histogram with the distribution curve overlaid – The frequency histogram assesses Normality of data. This is plotted with a distribution curve derived from the mean and the standard deviation of the data.
- Q-Q plot compares the two distributions and matches the quantiles of one with same quantiles of the other. These plots reveal the location, scale difference and identity of the outliers.
- The Shapiro-Wilk normality test: From the above methods, a graphical assessment can be made regarding normal or non-normal distribution of the data. However, the Shapiro's test will give a mathematical value as to whether or not normality exists.
- Linear Regression curves
- Spearman rank correlation test: Used to obtain the correlation with non-parametric data.
- Box plots: Graphical representations of the median and quartiles 1 and 3.
- Sensitivity analysis: A sensitivity analysis is performed to see if an outlier skews the data. In sensitivity analysis, the Spearman Rank correlation test is repeated after removing the outlier.
- Mann Whitney-U test: This is a non-parametric test equivalent to paired T-test. It is used to compare paired data. It uses the signs and the relative magnitudes of the data instead of the actual data. It calculates the difference and excludes zero. It arranges the remaining difference in the ascending order of magnitude, ignoring the signs and giving a rank number. The Null hypothesis states that there is no difference between the two groups.

- Coefficient of variation (CV): It gives a measure of the dispersion of all the points in a data around the mean of the data. It is calculated as CV = (Standard deviation/ Mean)
- Bias(% of the mean difference) = [(∑ Individual difference)/n]*100 , where,

  Individual difference = (Predicted – Measured)/Measured

  Reference: (83)
- Precision(% mean of absolute difference) = [(∑ Absolute of individual difference)/n]*100, where,

  Absolute of individual difference = (| Predicted – Measured |)/Measured

  Reference: (83)

Results

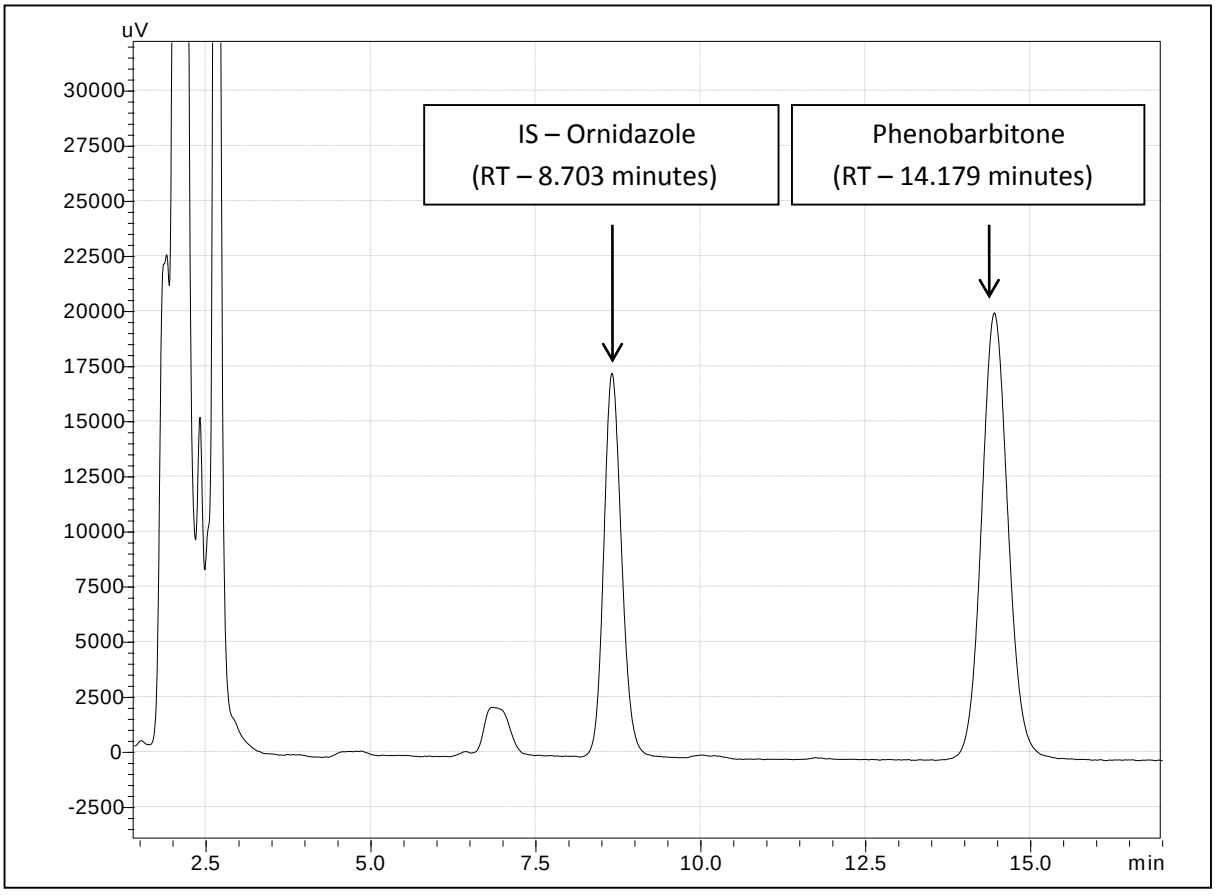
The results are presented in tabular and graphical format. The significance of the results is discussed in the following section.

Development of the assay for the measurement of phenobarbitone in serum

A) Water standards

From separate injections of the water standards, the retention times (RT) of Ornidazole (internal standard) and Phenobarbitone were 8.703 and 14.179 minutes respectively under the final standardised set of HPLC conditions.

Figure 12: Standard 40 µg/ml chromatogram showing the IS (Ornidazole) and Phenobarbitone peaks under the final standardised HPLC conditions (for serum)



B) <u>Blank matrix specimens</u>

Blank specimens from 10 different sources were analysed. The chromatogram traces of the blank specimens were compared against that for standard 5. No interfering peaks were seen under the retention times of both phenobarbitone and ornidazole.

Inferences due to the following antiepileptics were excluded -phenytoin, carbamazepine, valproate, oxcarbazepine, lamotrigine, levetiracetam, clonazepam, clobazam and topiramate.

Figure 13: Chromatogram showing the blank specimens of serum compared against standard 5

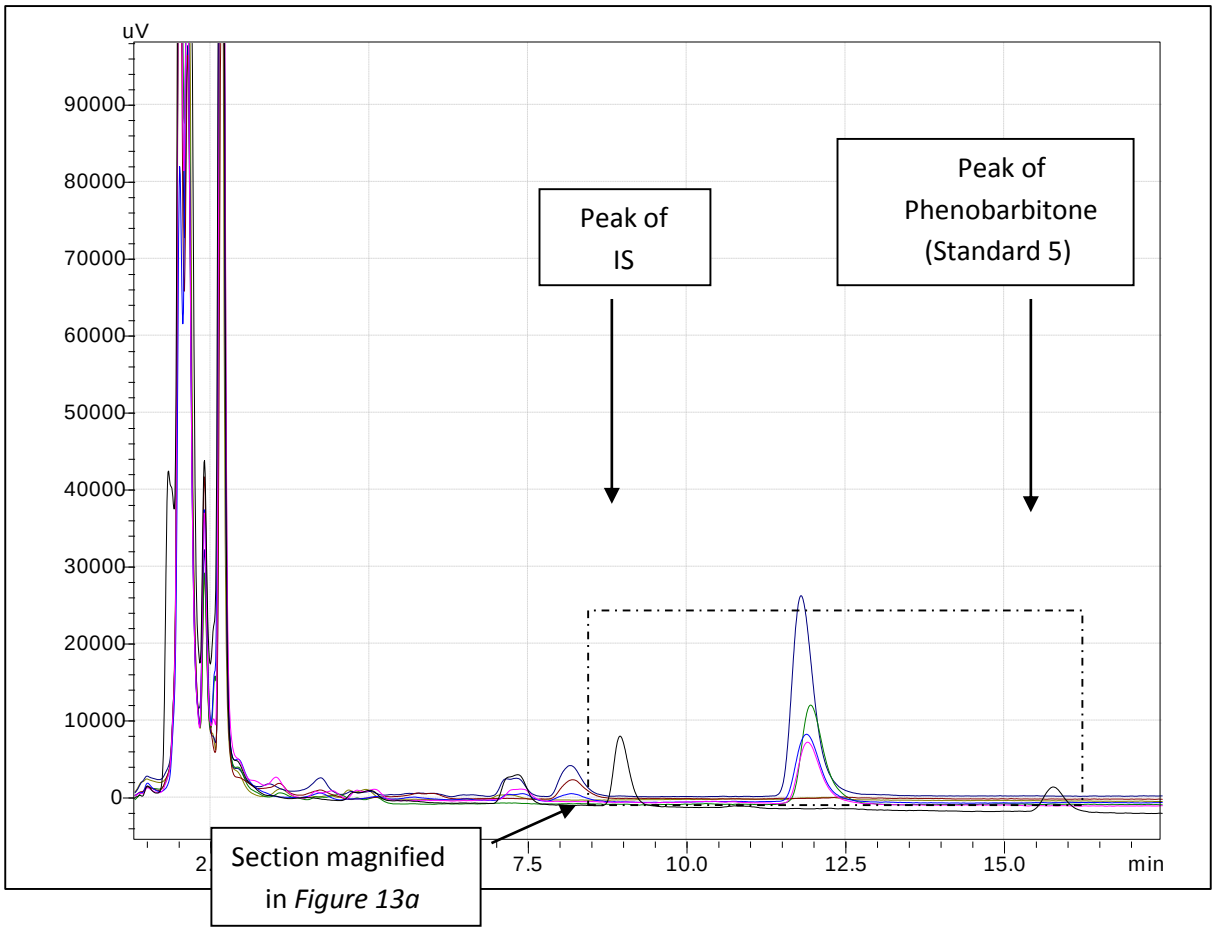


Figure 13a: Magnified view of the blank specimens compared against standard 5

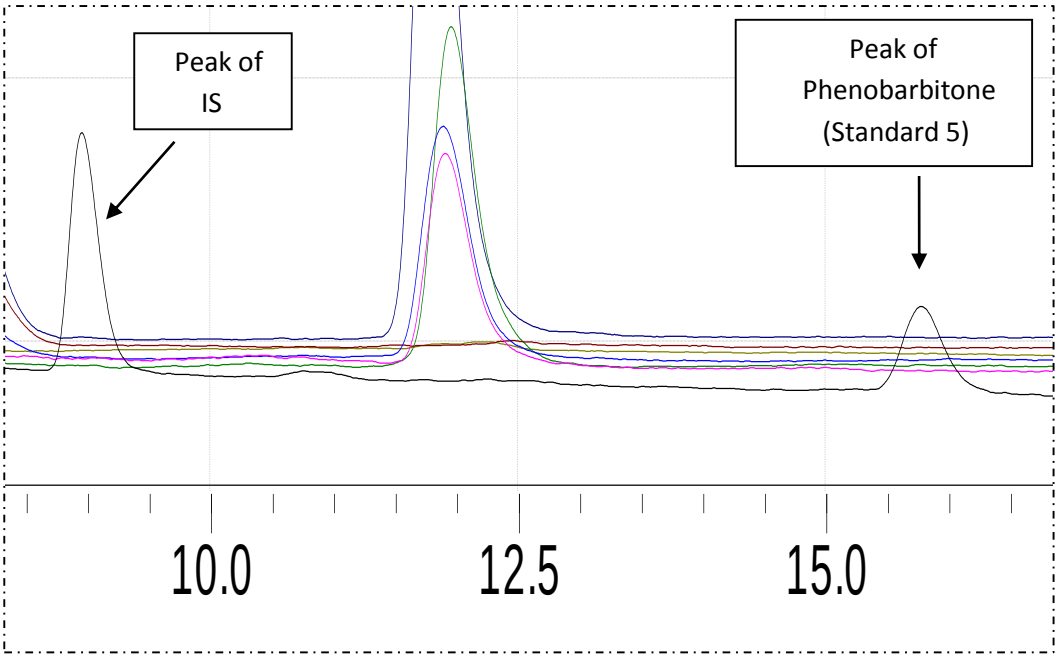


Table 2: Retention time and area under the curve of the IS (Ornidazole) and Phenobarbitone under the final standardised HPLC conditions (for serum)

	Internal Standard		Phenobarbitone		Ratio
Standard	RT	Area	RT	Area	
50	7.935	275182	13.783	955467	3.47
30	8.095	277026	14.045	588666	2.12
20	8.209	278566	14.237	375548	1.35
10	8.292	279970	14.363	190332	0.68
5	8.346	285070	14.476	91388	0.32
2.5	8.669	286804	15.259	38485	0.13
QC(15)	8.393	283633	14.555	286181	1.01
a =	- 0.034	**b =**	0.0704	**r =**	0.9998

C) Non-zero specimens

Specimens obtained from 10 different patients on phenobarbitone, were analysed to determine the expected range of concentrations in the study specimens. The range of concentrations obtained was between 2.5 to 50 µg/ml.

Figure 14: Chromatogram showing the non-zero specimens (for serum)

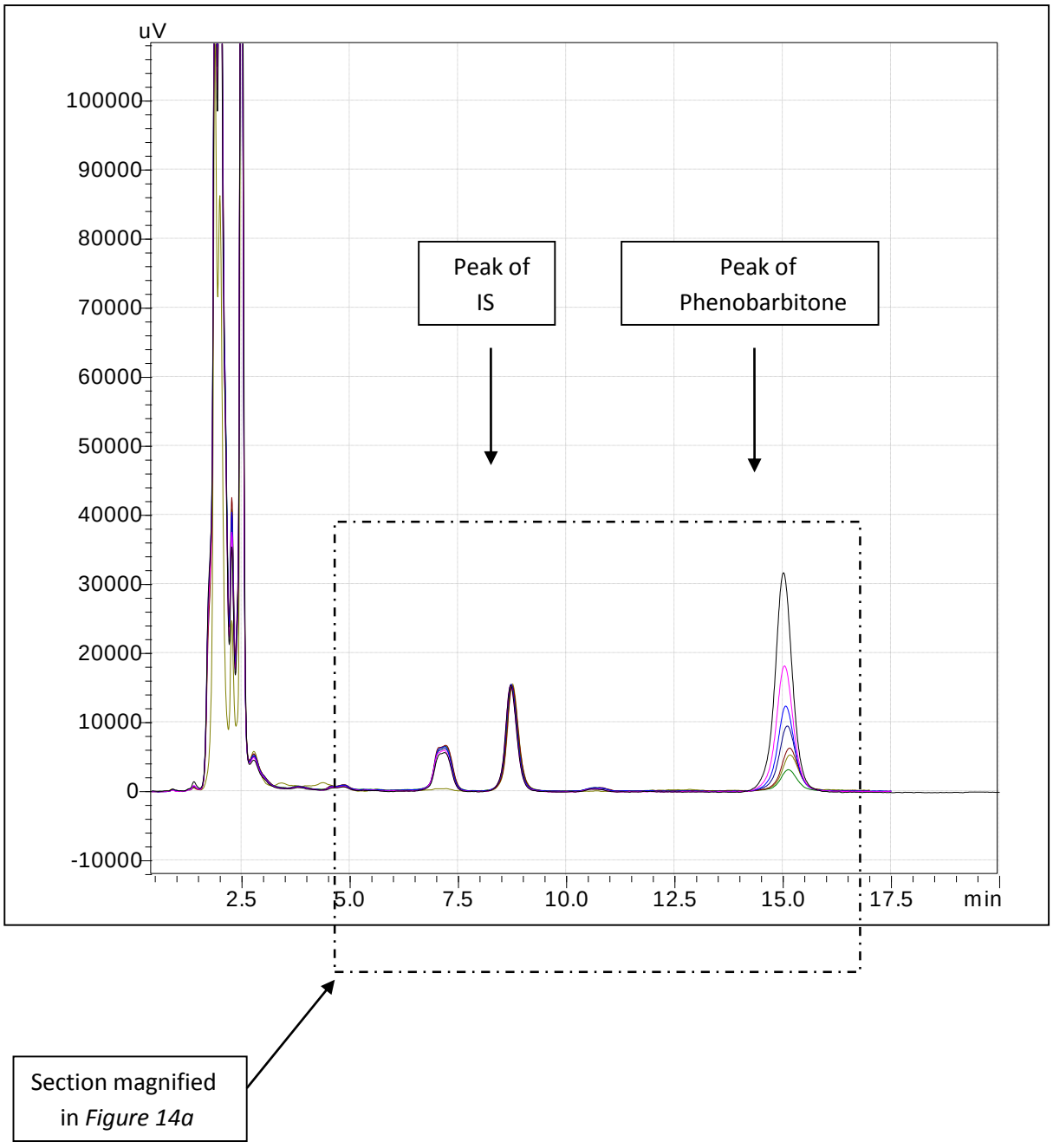
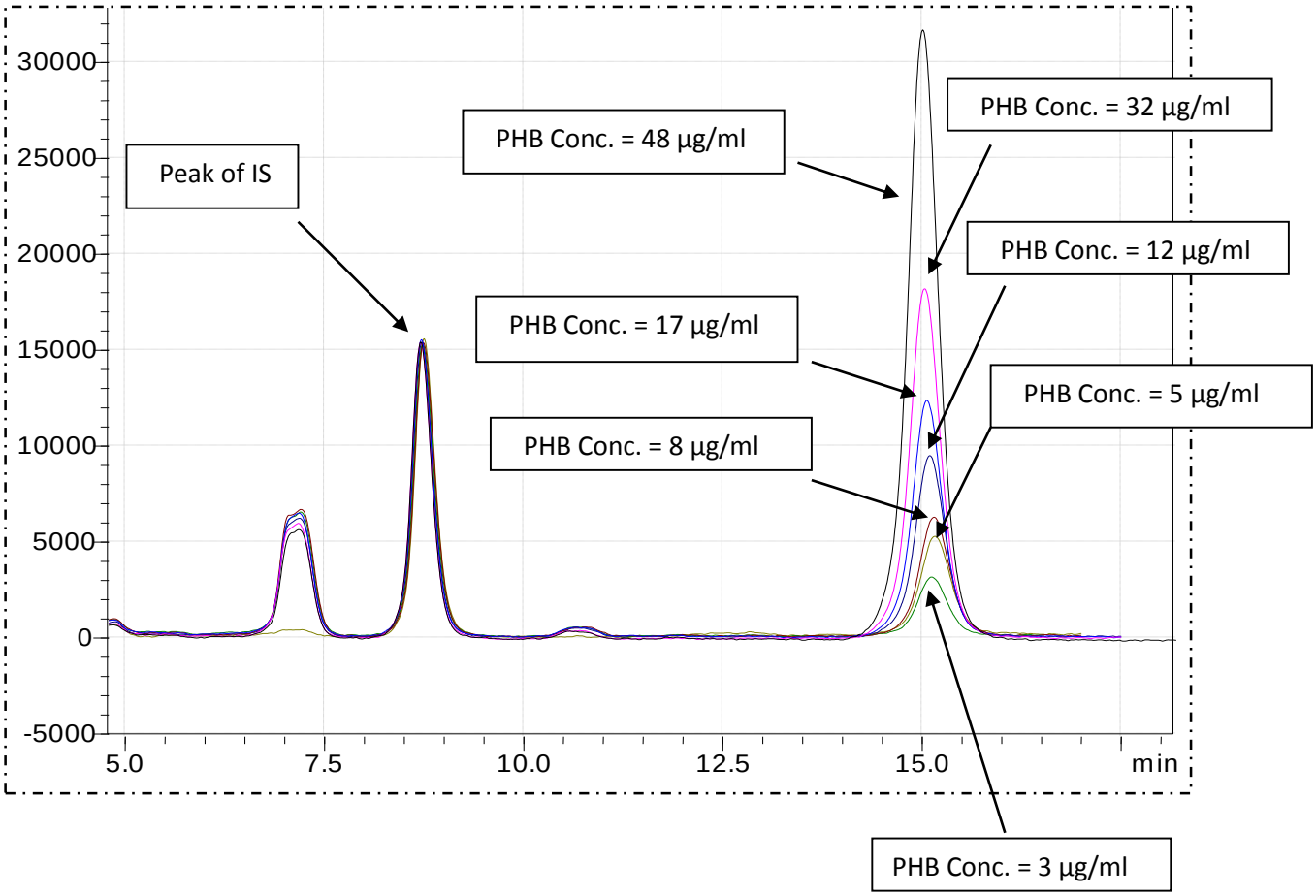


Figure 14a: Magnified view of the non-zero specimens (for serum)



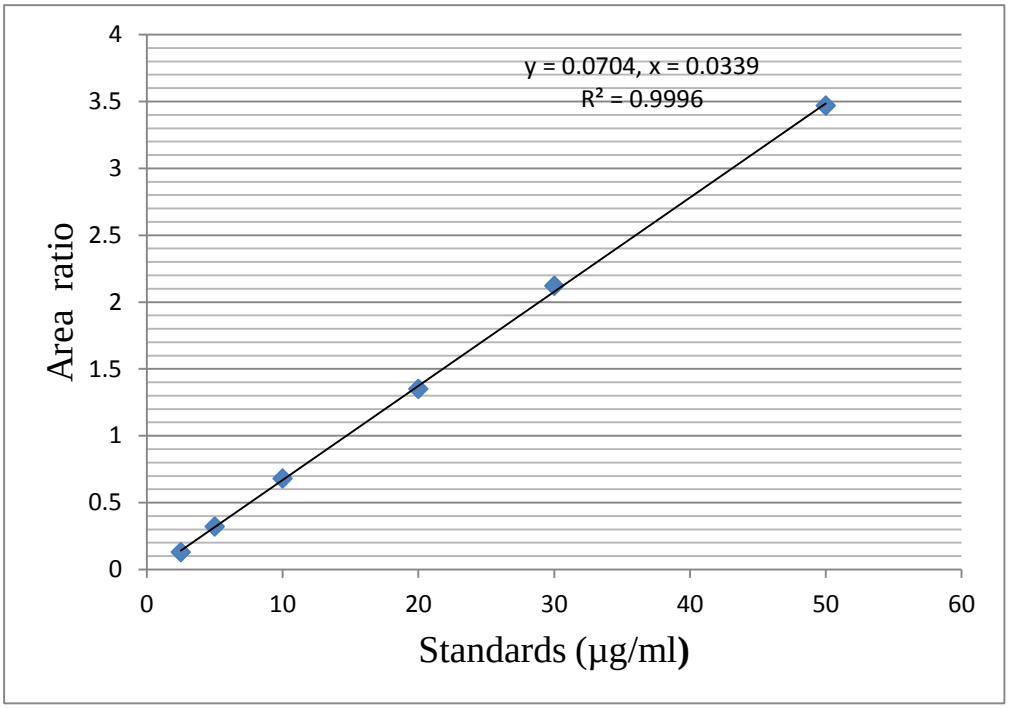
D) <u>Calibration curve</u>

Six calibrators – 50, 30, 20, 10, 5 & 2.5µg/ml were prepared from the standard stock solution and checked for linearity. The calibration curve was run 8 times. The linearity of the curve was maintained between 2.5 µg/ml and 50 µg/ml with a coefficient of correlation, r^2 = 0.9997.

Table 3: Statistical data showing linearity of the calibration curve (for serum)

Analyte	r^2 (mean ± S.D)	slope (mean ± S.D)	intercept (mean ± S.D)
Phenobarbitone	0.9997 ± 0.0001	0.0661 ± 0.0043	-0.0302 ± 0.0149

Figure 15: Linearity of the calibration curve (from 2.5 µg/ml to 50 µg/ml)



Final hplc conditions for the measurement of phenobarbitone in serum.

The final methodology is given in the Methodology Section on *pages 55 & 56*. A blank and standard chromatogram along with the area under the chromatogram curve and retention times is shown in *figure 12, 13.*

Validation of the assay for measuring phenobarbitone concentration in serum

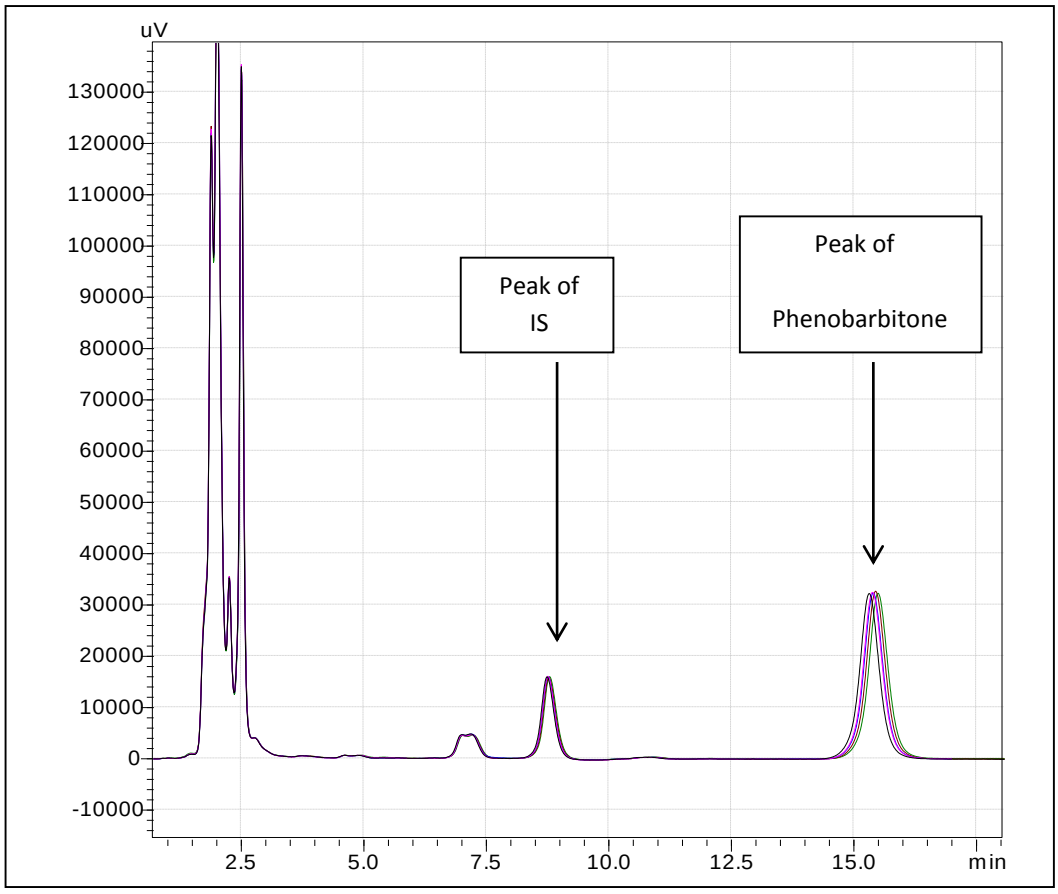
A) Intra-day variability (same batch, same day)

Intra - day variation of standards 50 and 5 are given in *table 4.* % CV was < 2% for both concentrations.

Table 4: Intra-day variation in the analysis of phenobarbitone from serum

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
50	50.72 ± 0.5227	1.03
5	5.37 ± .07155	1.33

Figure 16: Chromatogram for the Intra- day variation using standard 50 (n=5)



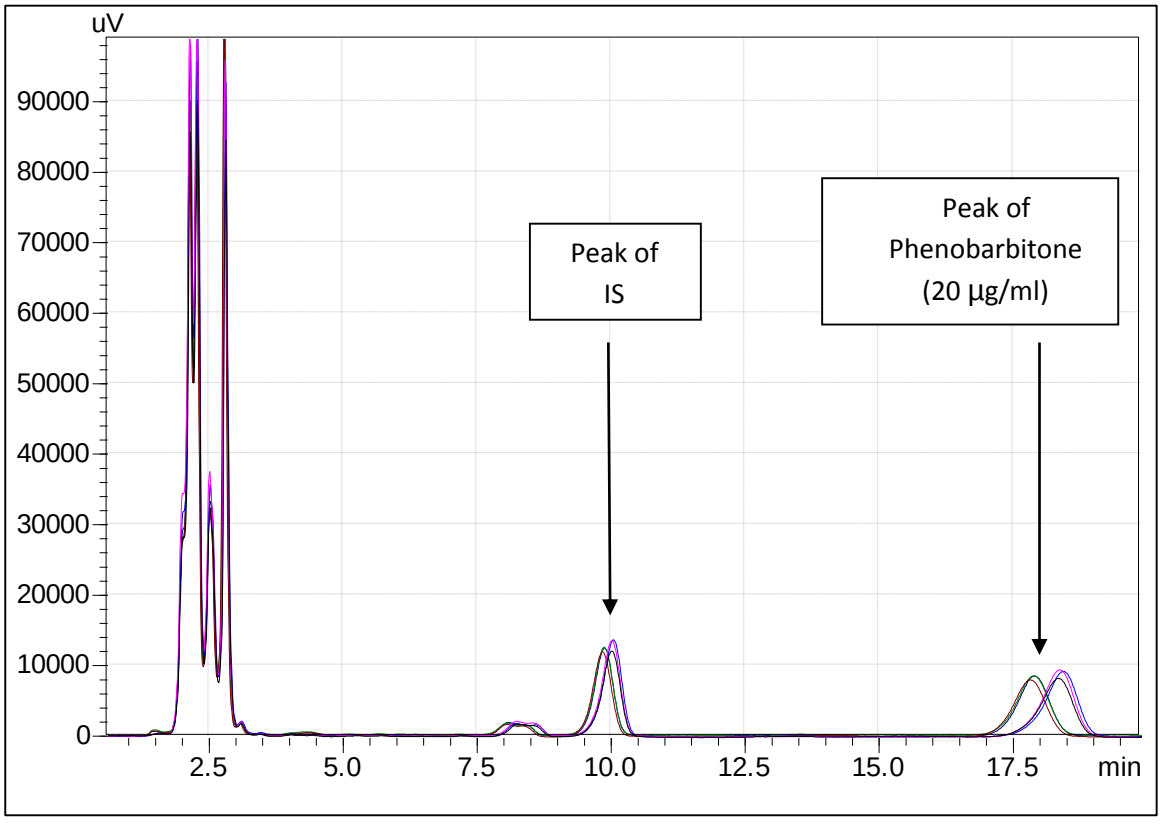
B) <u>Inter-day variability (same batch, different days)</u>

The results of the analysis of the 3 extractions of high, medium and low concentrations on the two separate days are given in *table 5*. The % CV was found to be < 4% for all three concentrations.

Table 5: Inter-day variation in the analysis of phenobarbitone from serum

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
50	48.96 ± 1.780	3.64
20	19.3 ± 0.4819	2.5
7	7.085 ± 0.2454	3.46

Figure 17: Chromatogram for the Inter-day variation using 20 µg/ml (n=3 on each day)



There is a small percentage shift in the retention times of the IS and the drug from day 1 to day 2. The % change in the retention time will be greater for an analyte which has a longer retention time, hence, the change in the retention time of the drug is more than that of IS.

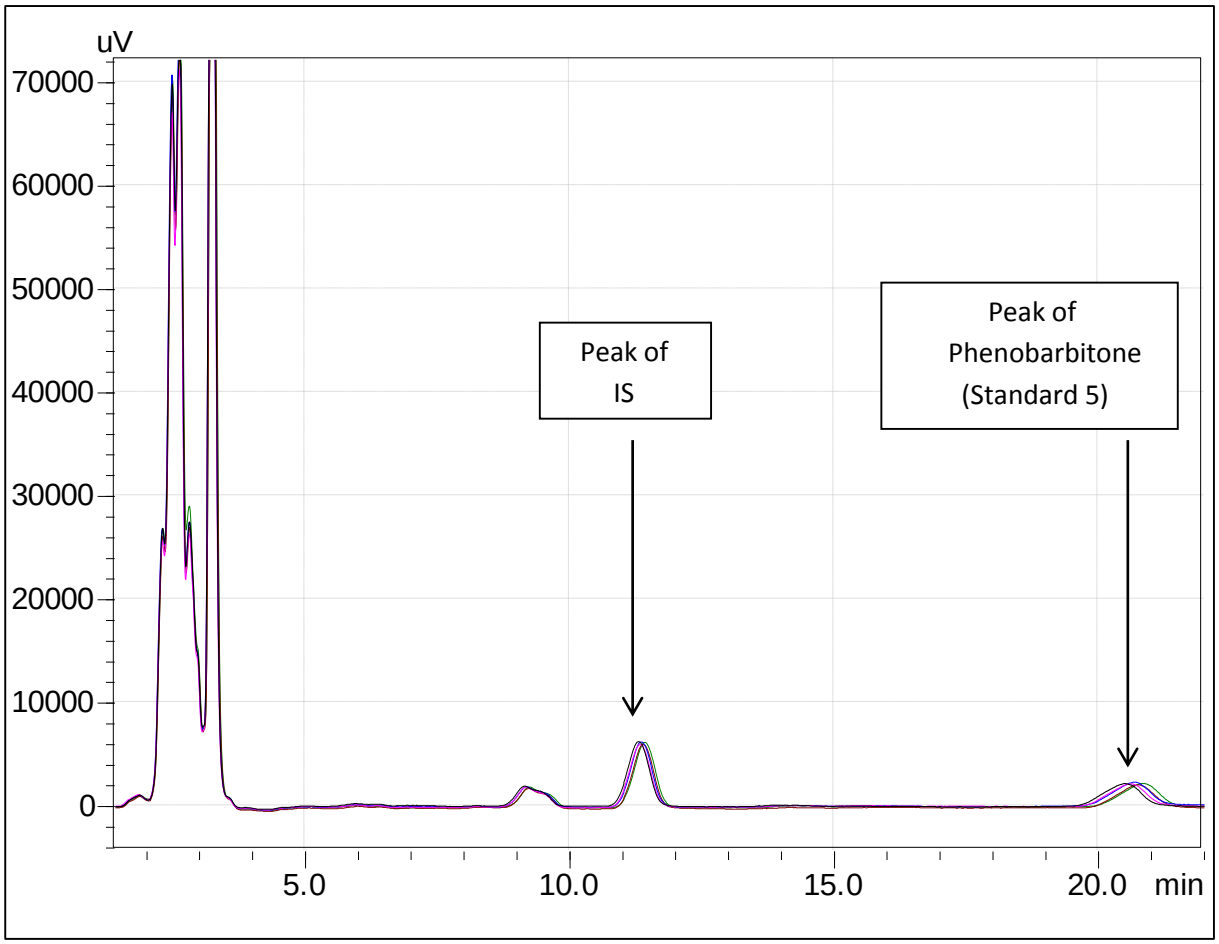
C) Reproducibility of injection

Evaluating the reproducibility of injection by analysing 5 repeat injections of a low and high concentration from the same extract, the % CV was found to be < 3% for both the low and high concentrations as shown in the table below.

Table 6: Reinjection reproducibility in the analysis of phenobarbitone from serum

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
50	49.156 ± 0.5763	1.17
5	5.106 ± 0.1289	2.53

Figure 18: Chromatogram showing reinjection reproducibility using Std 5 (n=5)

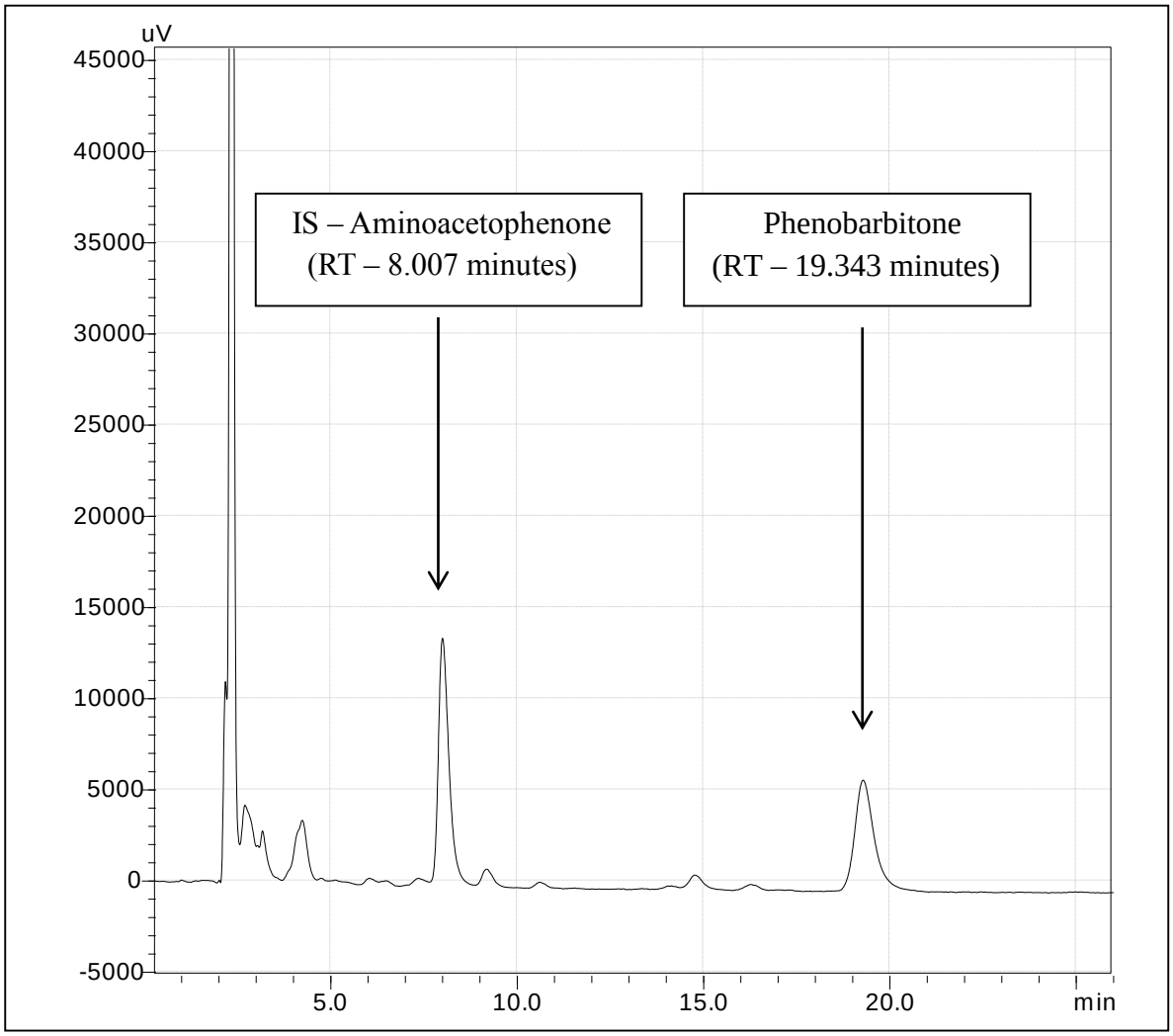


Development of the assay for the measurement of phenobarbitone in dried blood spot

A) Water standards

From separate injections of the water standards, the retention times of Aminoacetophenone (internal standard) and Phenobarbitone were 8.007 and 19.343 minutes respectively under the final standardised set of HPLC conditions.

Figure 19: *Standard 30 µg/ml chromatogram showing the IS (Aminoacetophenone) and Phenobarbitone peaks under the final standardised HPLC conditions (for dried blood spot)*



B) <u>Blank matrix specimens</u>

Blank specimens from 10 different sources were analysed. The chromatogram traces of the blank specimens were compared against that for standard 2.5. No interfering peaks were seen under retention times for both phenobarbitone and aminoacetophenone.

Inferences due to the following antiepileptics were excluded - phenytoin, carbamazepine, valproate, oxcarbazepine, lamotrigine, levetiracetam, clonazepam, clobazam and topiramate.

Figure 20: Chromatogram showing the blank specimens of dried blood spot compared against Standard 2.5

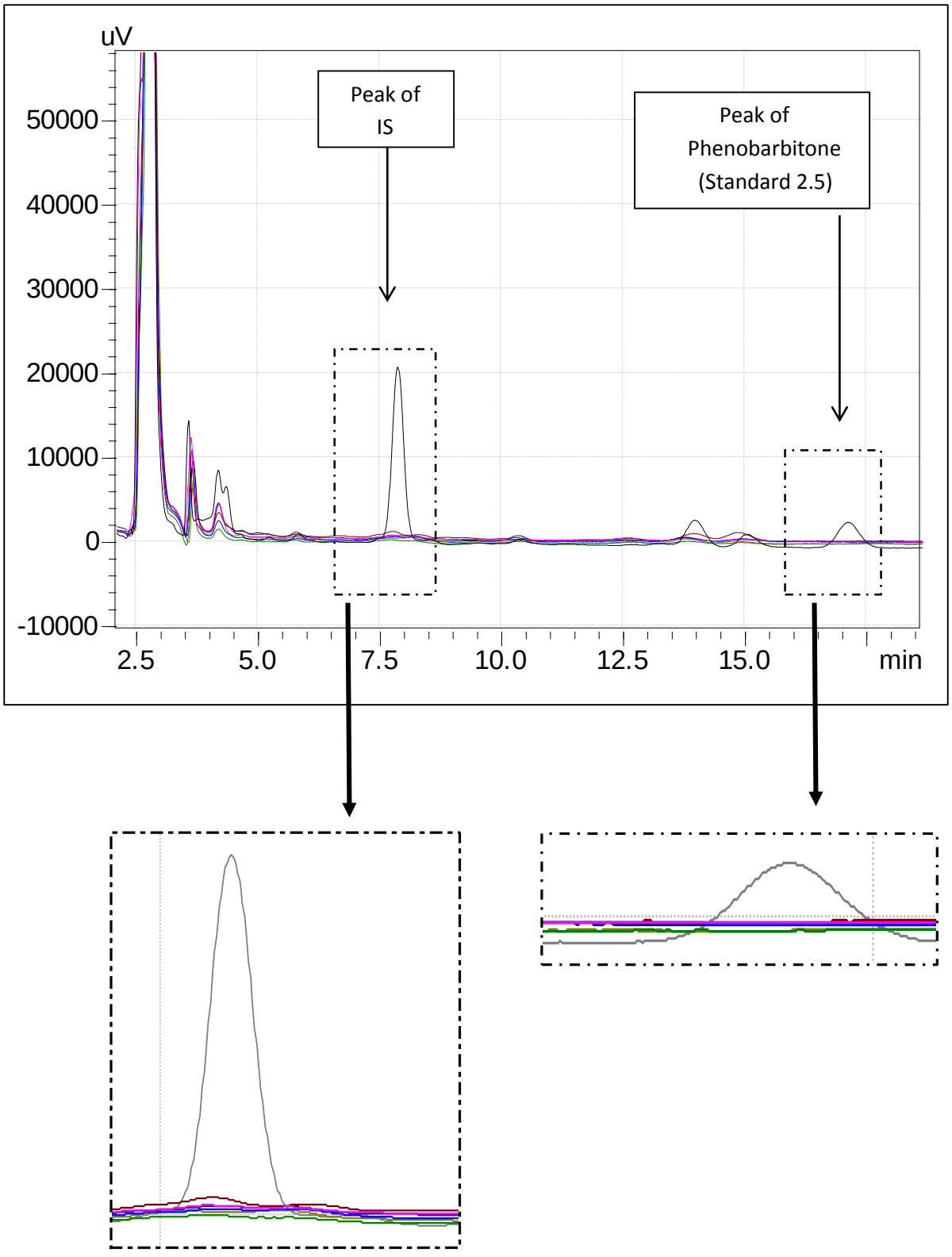


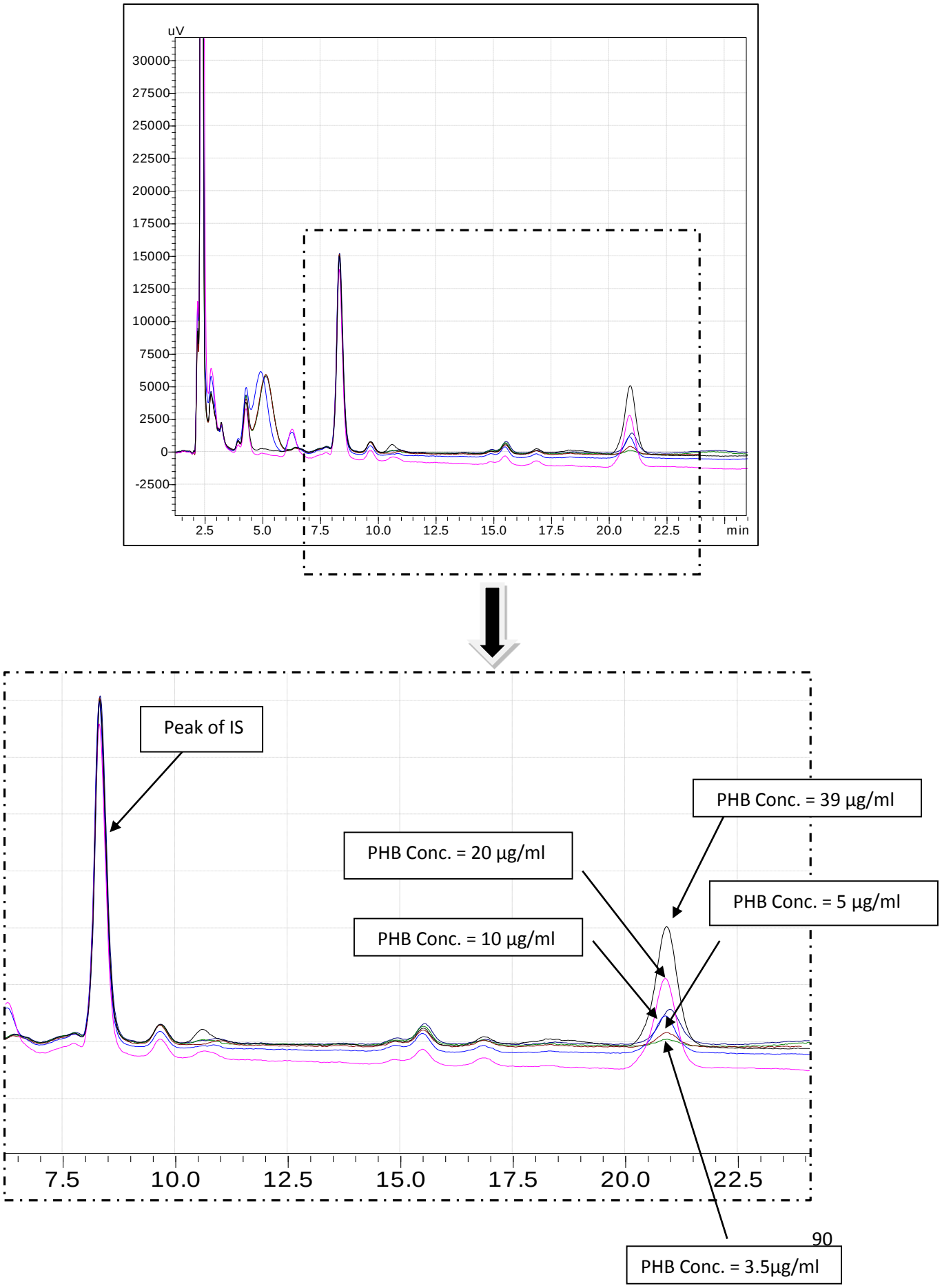
Table 7: Retention time and area under the curve of the IS (Aminoacetophenone) and Phenobarbitone under the final standardised HPLC conditions (for dried blood spot:

	Internal Standard		Phenobarbitone		Ratio
Std	RT	Area	RT	Area	
40	7.993	261356	19.275	222396	0.85
30	8.007	263205	19.343	170000	0.65
15	8.003	261750	19.299	85960	0.33
5	7.974	262803	19.087	33079	0.13
2.5	7.95	261893	19.068	18071	0.07
QC(10)	7.951	264245	19.038	62576	0.22
a = 0.0214		**b = 0.0208**		**r = 0.999**	

C) <u>Non-zero specimens</u>

Specimens obtained from 10 different patients on phenobarbitone were analysed to determine the expected range of concentrations in the study specimens. The range of concentrations obtained was between 2.5 µg/ml to 40 µg/ml.

Figure 21: Chromatogram showing the non-zero specimens (for dried blood spot)



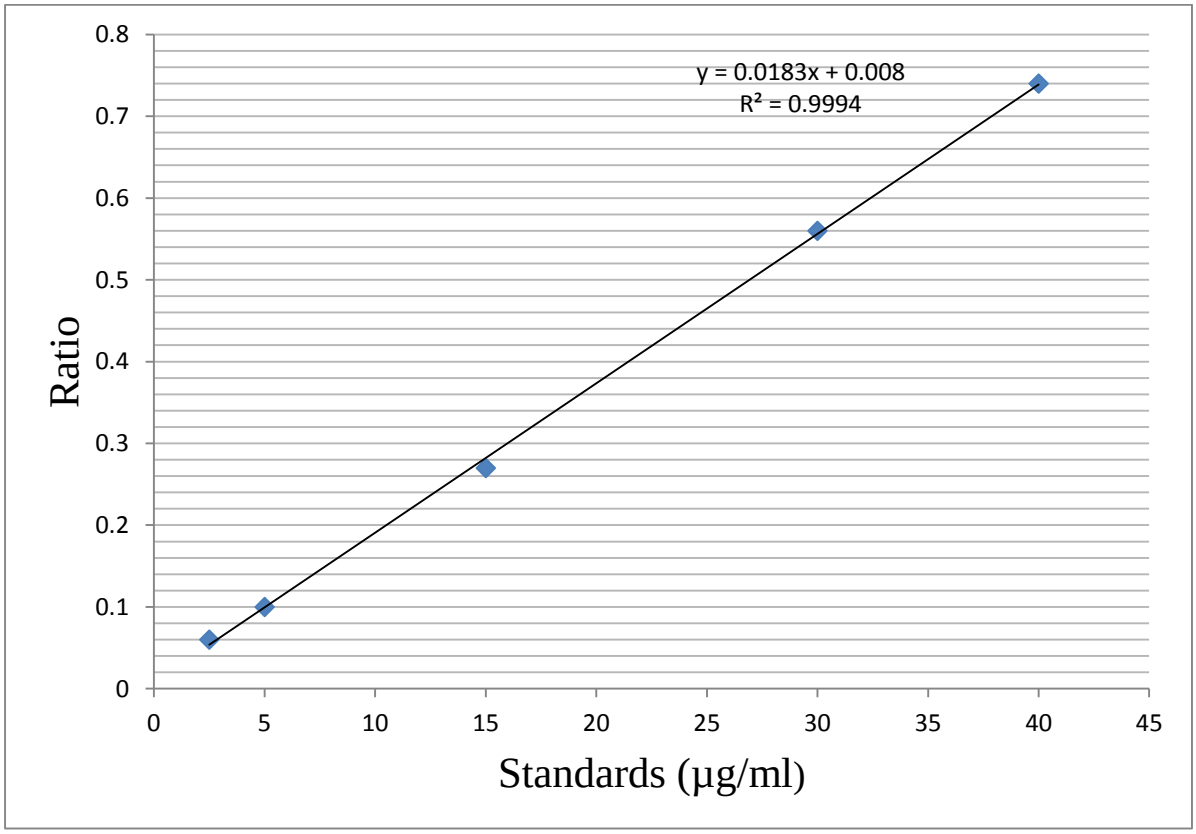
D) Calibration curve

Five calibrators – 40, 30, 15, 5 & 2.5µg/ml were prepared from the standard stock solution and checked for linearity. The calibration curve was run 8 times. The linearity of the curve was maintained between 2.5 µg/ml and 40 µg/ml with a coefficient of correlation, r^2 = 0.9973.

Table 8 : Statistical data showing linearity of the calibration curve (for dried blood spot)

Analyte	r^2 (mean ± S.D)	slope (mean ± S.D)	intercept (mean ± S.D)
Phenobarbitone	0.9973 ± 0.0013	0.0165 ± 0.0060	0.0030 ± 0.0117

Figure 22: Linearity of the calibration curve (from 2.5 µg/ml to 40 µg/ml)



E) Recovery

The mean recovery (%) was calculated as shown in the methodology section on *page 69* and was found to be 89.2%.

Table 9: % recovery of individual concentrations of phenobarbitone (from 2.5 µg/ml to 40 µg/ml)

Compound	Concentrations added (µg/ml)	Recovery (%)
Phenobarbitone	40	86.7
	30	94.8
	15	95.4
	5	79.4
	2.5	89.6
		Mean recovery = 89.2

Final hplc conditions for the measurement of phenobarbitone in dried blood spot

The final methodology is given in the Methodology Section on *page 63*. A blank and standard chromatogram along with the area under the chromatogram curve and retention times is shown in *figures 19 & 20*.

Validation of the assay for the measurement of phenobarbitone in dried blood spot

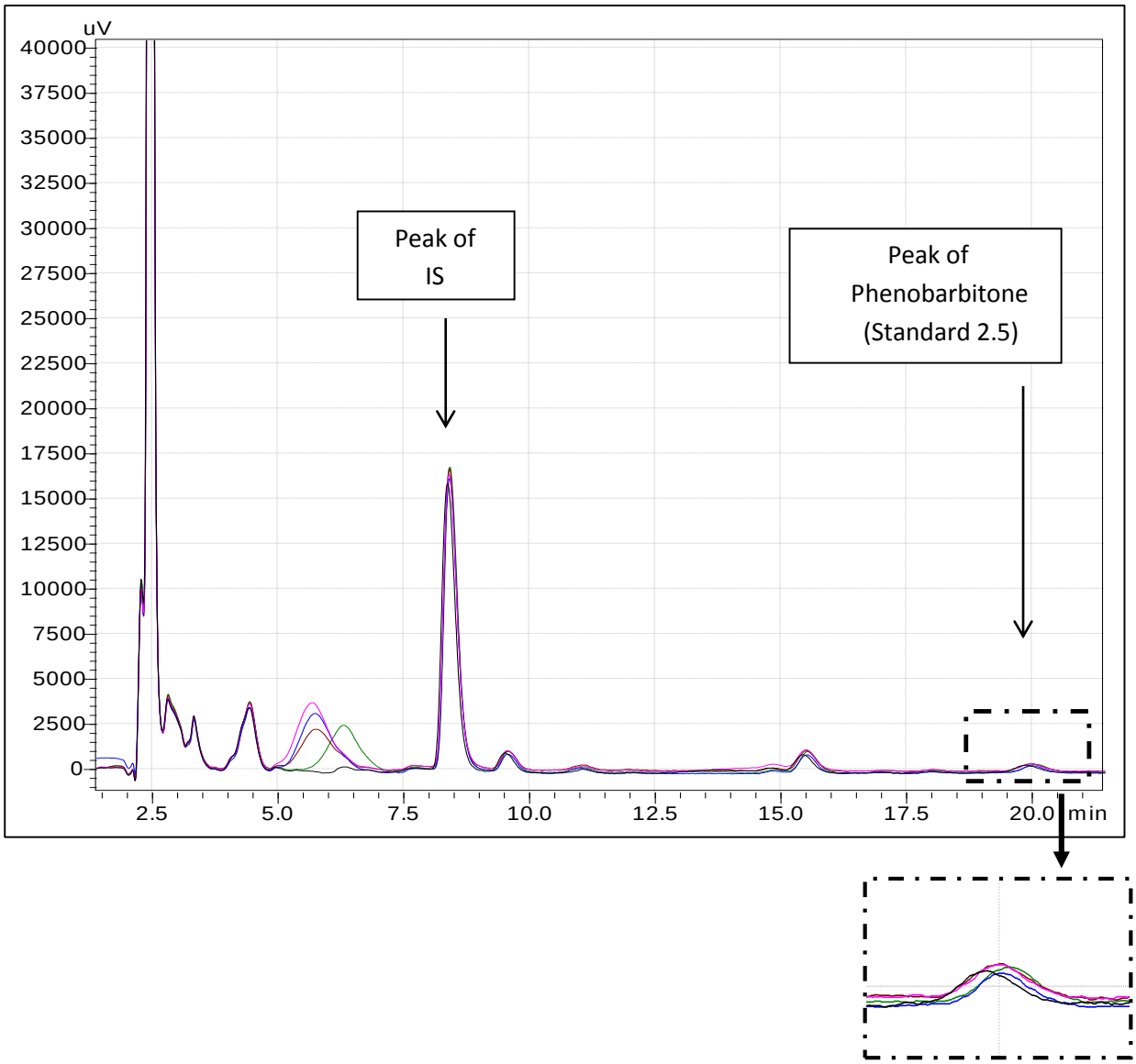
A) **Intra-day variability (same batch, same day)**

The % CV for the high and low concentrations is given below in *table 10*. The chromatogram is shown in *Figure 23.*

Table 10: Intra-day variation in the analysis of phenobarbitone from dried blood spot

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
40	35.844 ± 0.9073	2.53
2.5	3.016 ± 0.2684	8.9

Figure 23: Chromatogram showing Intra-day variation using Standard 2.5 (n=5)



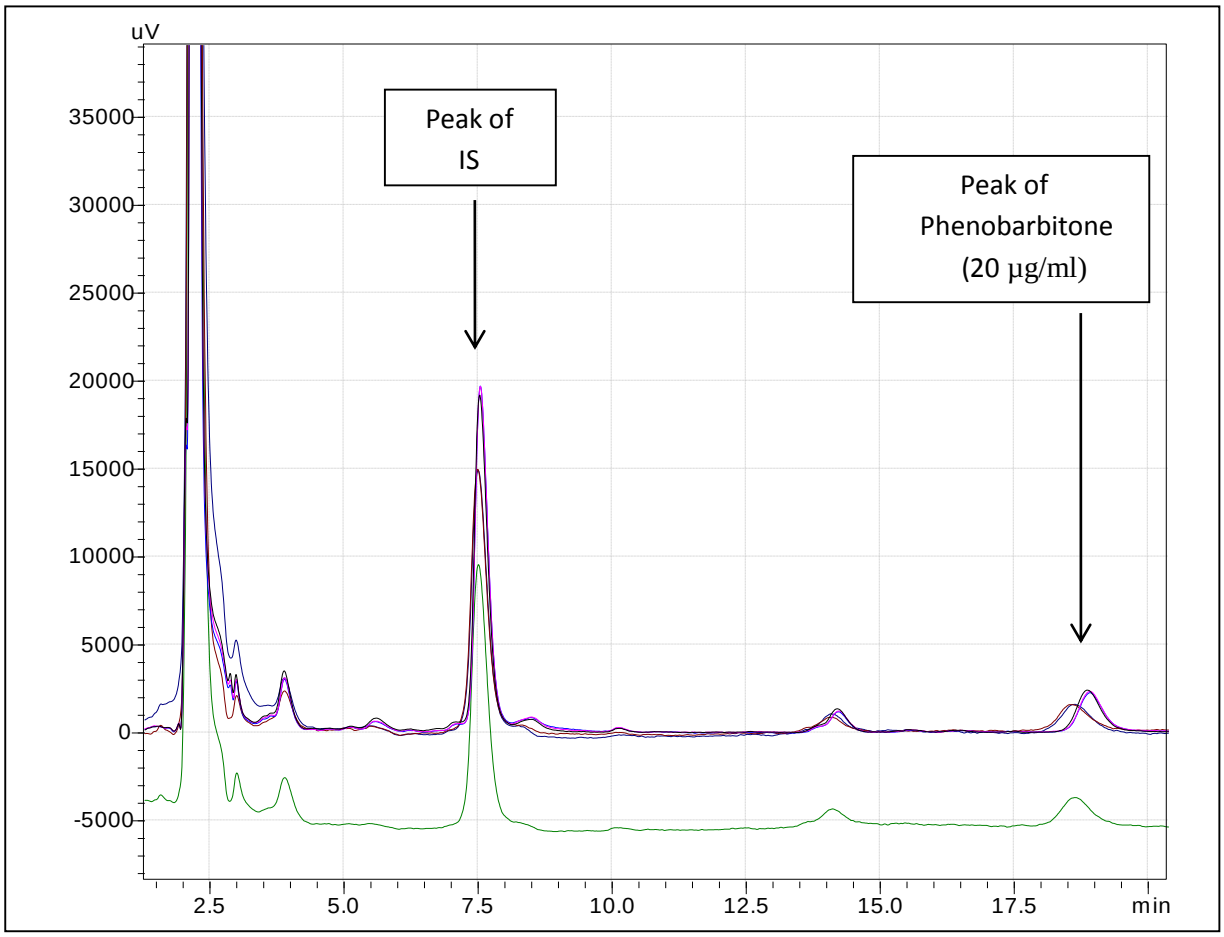
B) <u>Inter-day variability (same batch, different days)</u>

Table 11 gives the % CV for the inter - day variation of the high, medium and low concentrations on the two separate days.

Table 11: Inter-day variation in the analysis of phenobarbitone from dried blood spot

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
35	35.933 ± 1.1321	3.15
20	20.573 ± 0.8426	4.1
7	7.4033 ± 0.4856	6.56

Figure 24: Chromatogram showing Inter-day variation using 20 µg/ml (n=3 on each day)



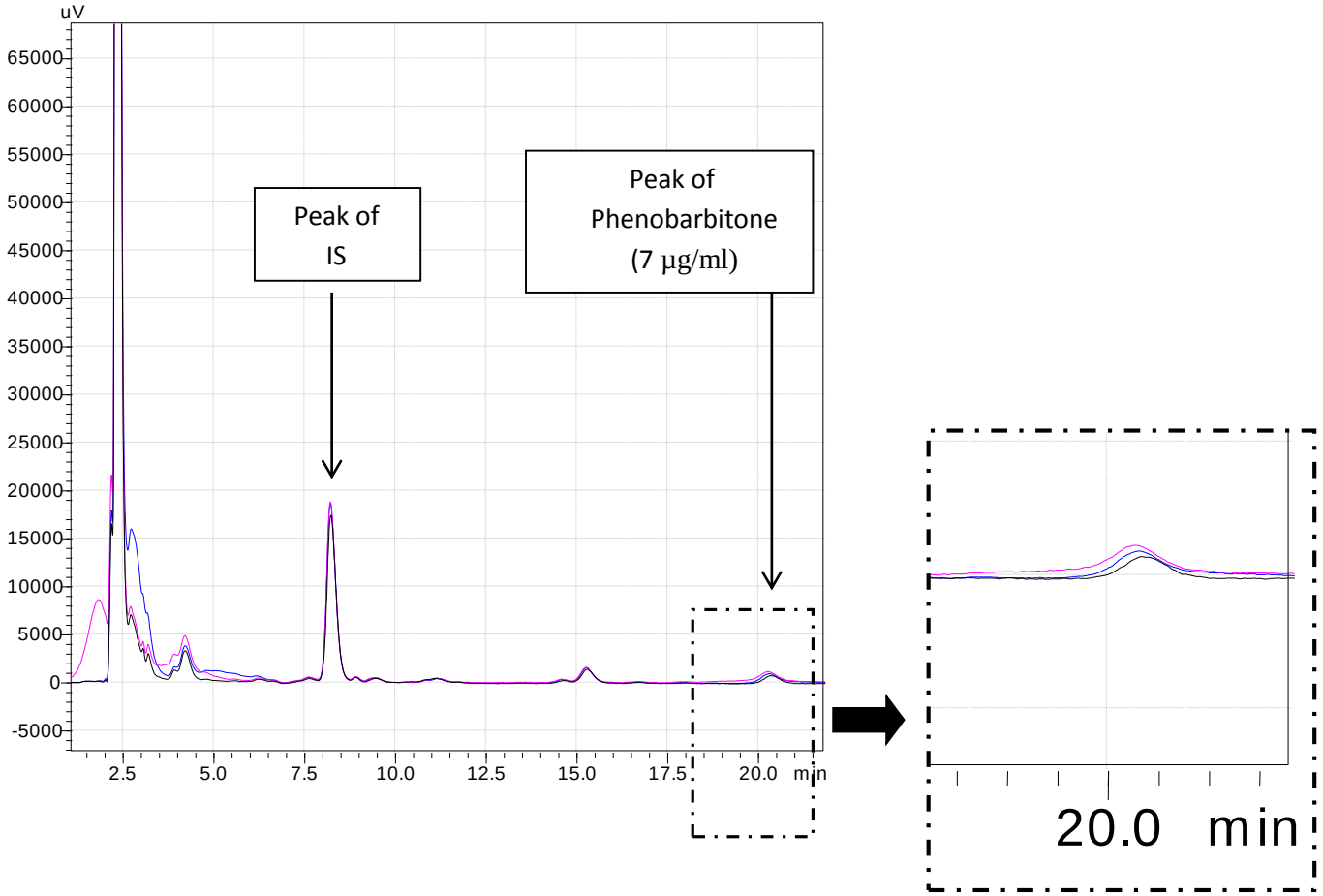
C) <u>Reproducibility of injection</u>

The reproducibility of injection evaluated using 3 repeat injections of a low, medium and high concentration, gave a % CV of < 2% for all the concentrations.

Table 12: Reinjection reproducibility in the analysis of phenobarbitone from dried blood spot

Concentration added (µg/ml)	Concentration measured mean ± S.D (µg/ml)	%CV
35	32.78 ± 0.00	0
20	19.7167 ± 0.4619	2.3426
7	6.7300 ± 0.00	0

Figure 25: Chromatogram showing Reinjection reproducibility using 7 µg/ml (n=3)



D) Stability

Phenobarbitone was found to be stable up to the 26 days tested in a DBS specimen, both under laboratory and simulated transport conditions. Refer *table 1 – 4* under the Appendix section for details.

Validation completed.

The above validated assays were then applied to the measurement of phenobarbitone in the serum and dried blood spots of study patients.

Study patients:

The R – program (version 3.1.5) was used for the statistical analysis and graphical representations. The script for each of the relevant programs is shown under the appropriate heading in a box.

1. The mean, median and Interquartile ranges for the concentration of PHB in serum and the DBS concentration were determined using the R-program

* R script for the above (# *signifies a comment*):

```
attach(Final_pts)        # This attaches the relevant file and must be performed at the
                         # start of each run.  Final_pts is the csv file with the serum
                         # concentrations and the CDBS concentrations (Refer
                        # Appendix – table 5)

summary(Final_pts)       # To get a summary of the data
```

Table 13: Summary data of the study patients (n=36)

I.D.	Serum Concentration	Corrected DBS Concentration	Difference between Corrected DBS & Serum Concentration	Mean of Corrected DBS & Serum Concentration
Mean	19.68	20.49	0.8103	20.09
Median	17.13	16.29	-0.035	16.72
Minimum	2.41	2.2	-2.69	2.3
Maximum	58.14	63.36	7.59	60
1st Quarter	10.15	10.24	-0.7	10.21
3rd Quarter	28.67	28.98	1.53	29.15

Demographic details such as age and gender, and co-medications are shown in *table 14.*

Table 14: *Distribution of age, gender and concomitant medications*

	Males	Females	Age (median & range)	+ PHT	+ PHB	+ Val	+ CBZ	+ LTG	+ LVT	+ Clobazam	+ Clonazepam	+ Other
N	22	14	39 (18-61)	14	36	4	10	1	Nil	5	1	20

*PHT – Phenytoin, PHB – Phenobarbitone, Val – Valproate, CBZ – Carbamazepine, LTG – Lamotrigine, LVT – Levetiracetam, Other medications (Folic acid - 9, Liv-52 - 1, Olanzapine - 1, Glyciphage - 1, Glimepiride - 1, Amitriptyline - 1, L-Carnitine - 1, Coenzyme – Q - 1, Riboflavin - 1,Salbutamol - 1, Amlodipine - 1, Aspirin - 1)

2. Calculate the precision and the bias between the predicted and the measured serum concentration. (Before and after correcting for PCV)

From the equations shown on *page 75* the following results were obtained.

Before correcting for PCV i.e. uncorrected DBS (UDBS) - Imprecision = 10%

Before correcting for PCV i.e. uncorrected DBS (UDBS) - Bias = 1%

After correcting for PCV i.e. corrected DBS (CDBS) - Imprecision = 8%

After correcting for PCV i.e. corrected DBS (CDBS) - Bias = 0.49%

All subsequent calculations were based on CDBS

3: Bland Altman Plot

It shows the mean and standard deviation of the mean of the difference (*Figure 26*)

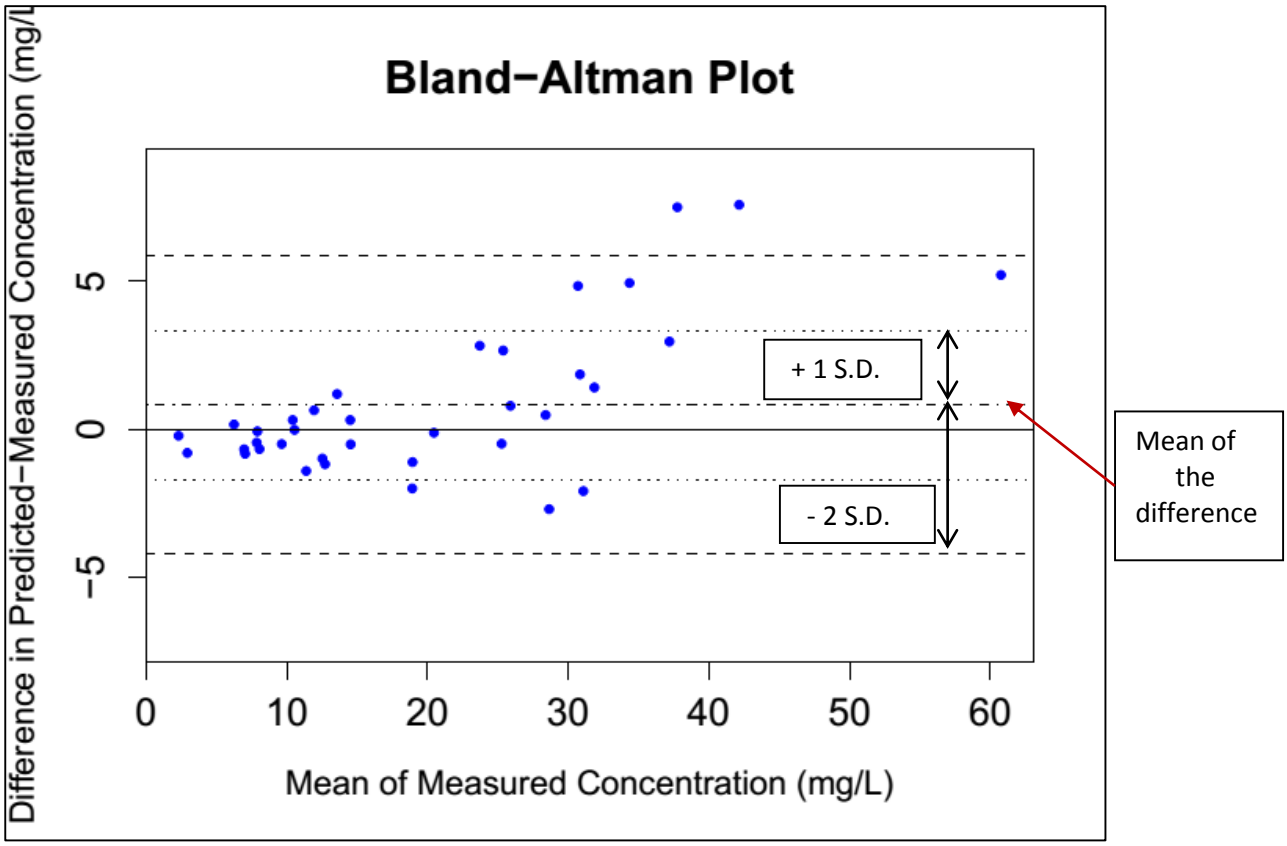
* R script for the above (# signifies a comment):

```
attach(Final_pts)

# Bland-Altman plot R function (Based on the program by: jmmateos@mce.hggm.es)

means <- mean   # <- is a symbol meaning assign to

diffs <- diff

mdiff <- mean(diffs)

sddiff <- sd(diffs)
```

R Script continued on next page.

```
# Compute the figure limits

ylimh <- mdiff + 2 * sddiff                    #Upper limit on y axis is +2 standard deviations

yliml <- mdiff - 2 * sddiff            #Lower limit on y axis is - 2 standard deviations

# Plot data

plot(diffs ~ means, pch=20, col="blue", main="Bland-Altman Plot",xlab="Mean of Measured Concentration (mg/L)",

cex.lab=0.9, ylab ="Difference in Predicted-Measured Concentration (mg/L)",

ylim = c(yliml-4, 12))

# Explanation of symbols,' ~' symbol means plot differences and means against each other,

# pch=format for the symbol = full spot, 'main' is for the graph title, 'col' assigns a colour

# xlab and ylab ares the labels for the x and y axis respectively, ylim is the full y axis.

abline(h = mdiff,lty=4)      # Sets the centre line, lty is the line type

abline(h=0)                          # Sets a line at 0

abline(h = mdiff + 1 * sddiff, lty = 3)      # shows a line at 1 standard deviation

abline(h = mdiff - 1 * sddiff, lty = 3)

abline(h = mdiff + 2 * sddiff, lty = 2)                # shows a line at 2 standard deviations

abline(h = mdiff - 2 * sddiff, lty = 2)
```

Figure 26: Bland Altman plot showing the mean of the difference between the predicted and the measured serum concentrations (in study patients)



From the Bland Altman plot, the mean of the difference between the predicted and the measured concentration was 0.81, with a standard deviation of 2.5.

4: Test for normal distribution of data in the study patients

The Q-Q plot and the histogram with the normal distribution curve based on the mean and the standard deviation of the data is shown below in *figures 27-30.* From both the Q-Q plot and the histogram with the normal distribution curve, the data for serum and corrected dried blood spot concentrations do not appear normally distributed.

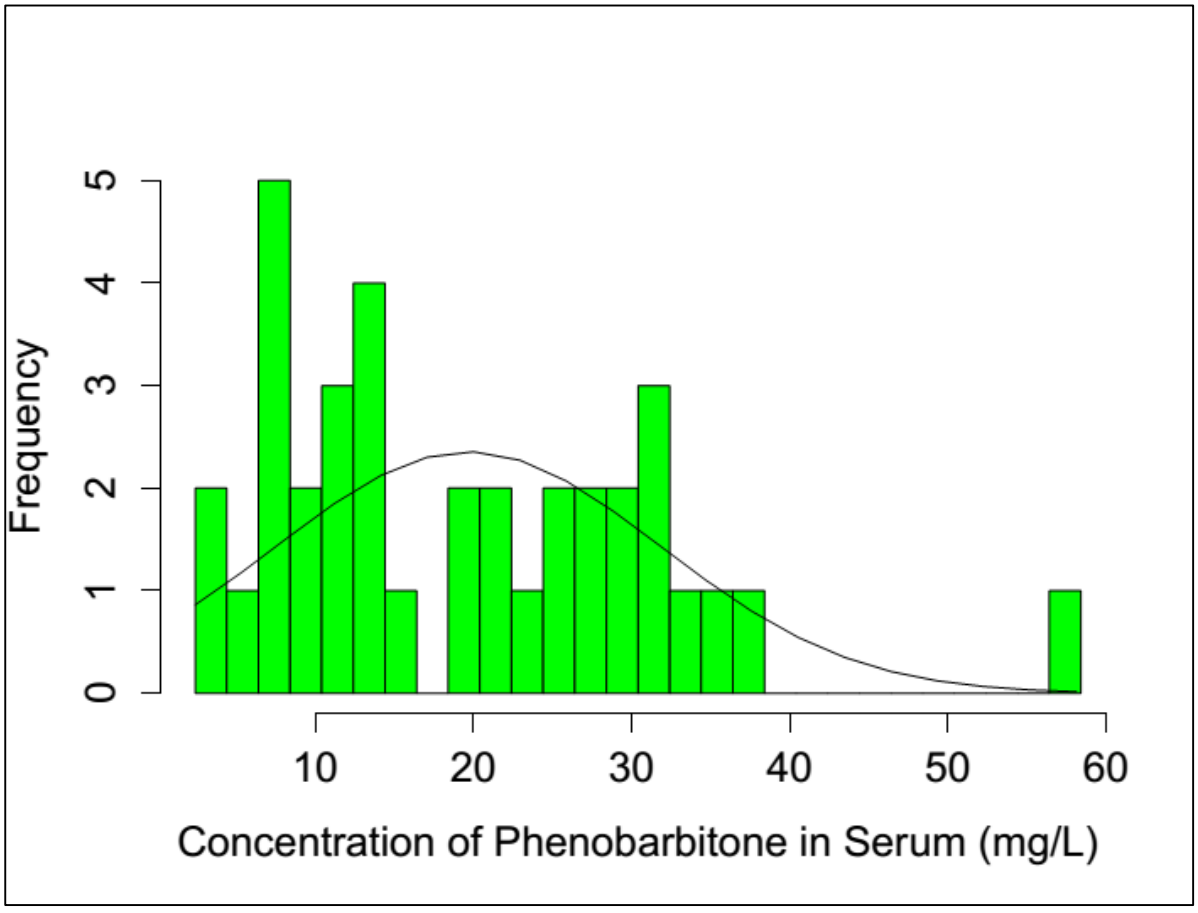
* R script for the above (# signifies a comment):

```
attach(Final_pts)            # This attaches the relevant file

min(Serum.Conc.)             # Find the minimum concentration

max(Serum.Conc.)             # Find the maximum concentration

# To plot the histogram.

bins=seq(min(Serum.Conc.),max(Serum.Conc.)+2,2)     # bins is a single bar on the
                                                    # histogram

hist(Serum.Conc.,breaks=bins, main="",ylab="Frequency.", xlab="Concentration of
Phenobarbitone in Serum (mg/L)", col="green")

# main: main heading, ylab and xlab refer to the y and x labels, col is the colour

# Putting in a distribution curve:

# 1st step: Assign our histogram to h

h<-hist(Serum.Conc.,breaks=bins,main="",ylab="Frequency",xlab="Concentration of
Phenobarbitone in Serum (mg/L)",col="green")

# <- this symbol means assign the statement to for example in this case the letter h

# 2nd step:  Create 20 bins from our data – which helps define the data better.

xfit<-seq(min(Serum.Conc.),max(Serum.Conc.),length=20)
```

R Script continued on next page

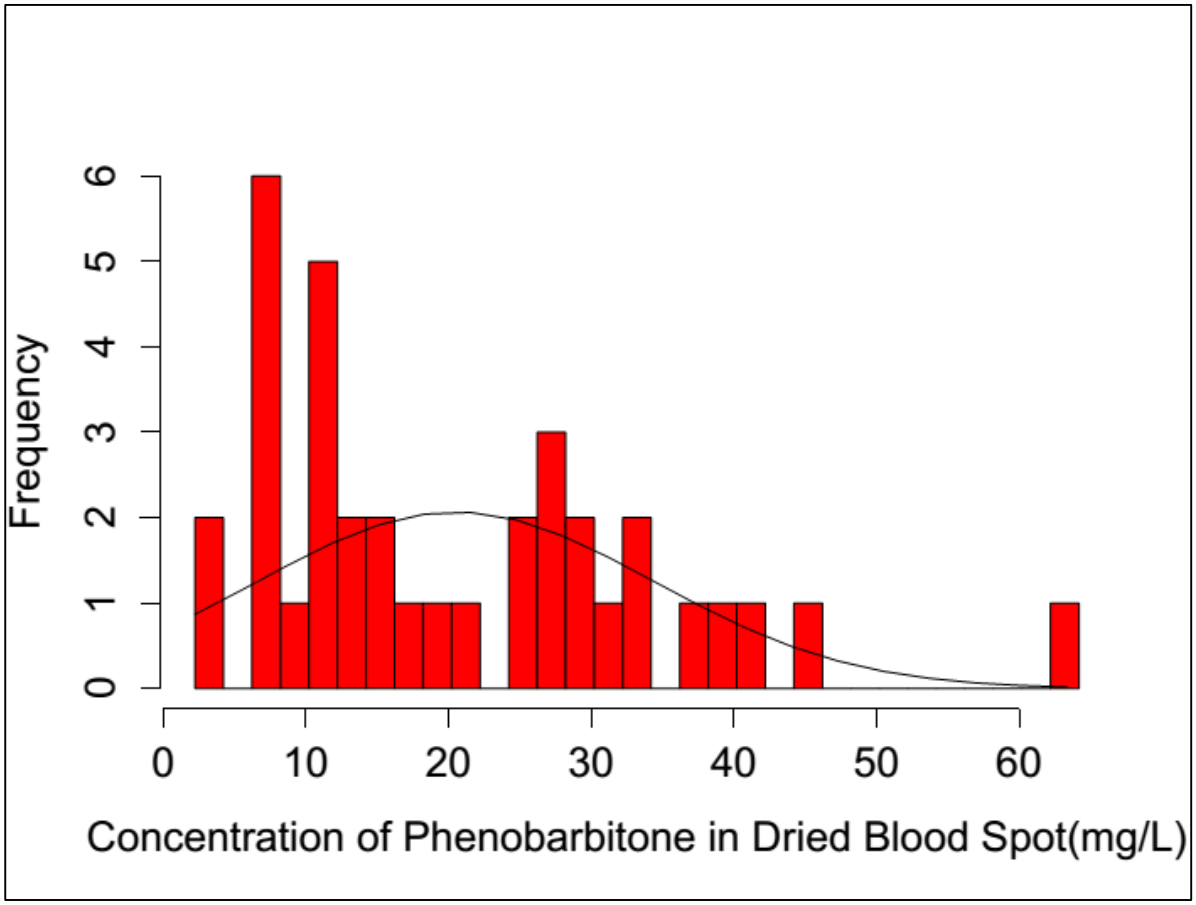
```
# 3rd step:  Given our data mean and sd , find the normal distribution

yfit<-dnorm(xfit,mean=mean(Serum.Conc.),,sd=sd(Serum.Conc.))

# 4th step:  Fit the normal distribution to our data

yfit<-yfit*diff(h$mids[1:2])*length(Serum.Conc.)

# 5th step:  Plot these lines lwd= line width, 1 is default, 2 is double 1

lines(xfit,yfit,lwd=1)
```

Figure 27: Histogram with Normal distribution curve – Serum Concentration



This was also performed for the Corrected dried blood spot concentration and this is shown in the figure below:

Figure 28: Histogram with normal distribution curve – Corrected Dried blood spot concentration

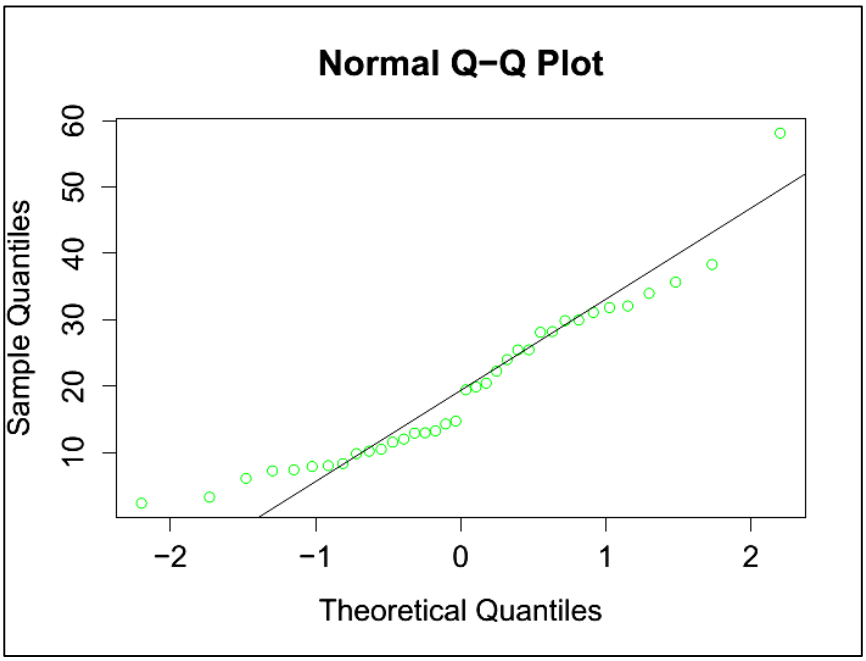


Q-Q plot

* R script for the above (# signifies a comment):

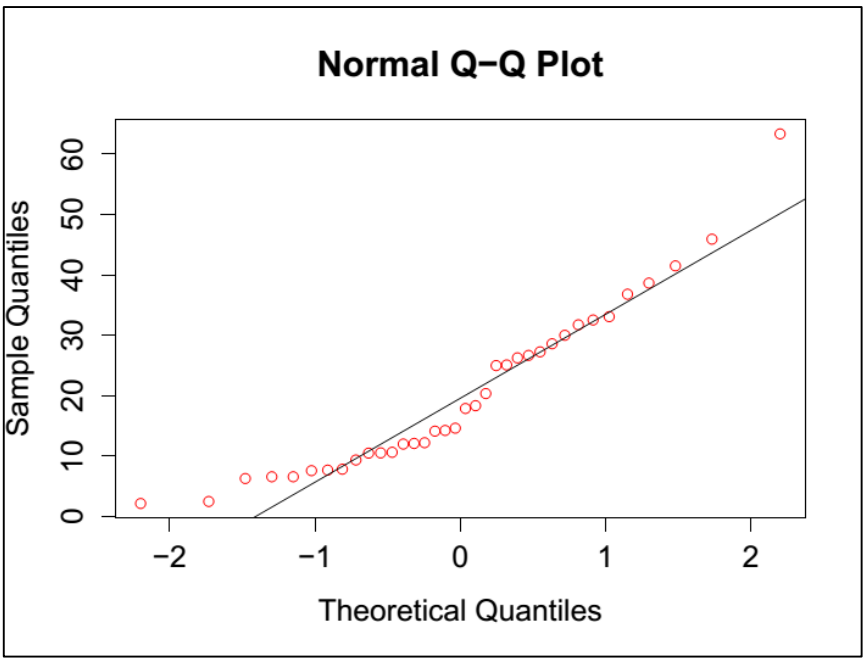
```
attach(Final_pts)
qqnorm(Serum.Conc.,col="green")          # Code for a normal Q-Q plot:
qqline(Serum.Conc.,col="black")
```

Figure 29: Q-Q plot of Serum Concentration



This was also performed for the Corrected dried blood spot concentration and this is shown in the figure below.

Figure 30: Q-Q plot of Corrected Dried blood spot concentration



5: The Shapiro-Wilk Normality test for statistical determination of normality

* R script for the above (# signifies a comment):

```
attach(Final_pts)

# To test for normality, we apply the Shapiro test

shapiro.test(Serum.Conc.)

shapiro.test(DBS.Conc.)
```

The assumption and null hypothesis for this test is that the data are normally distributed. On performing the test a 'W' value is returned. There is a Shapiro-Wilk table which gives the p-values corresponding to the 'W' value and the number of patients in the test. The W and P value of the Serum Concentration (SC) and the Corrected Dried blood spot Concentration (CDBS) obtained by the Shapiro test as given in *Table 15.*

Table 15: Results of the Shapiro-Wilk normality test

Data	W	P. value
Serum Concentration	0.9241	0.01664
Corrected DBS	0.9172	0.01041

Thus, from the Shapiro-Wilk normality test, it was confirmed that the data is not normally distributed in the serum and corrected dried blood spot.

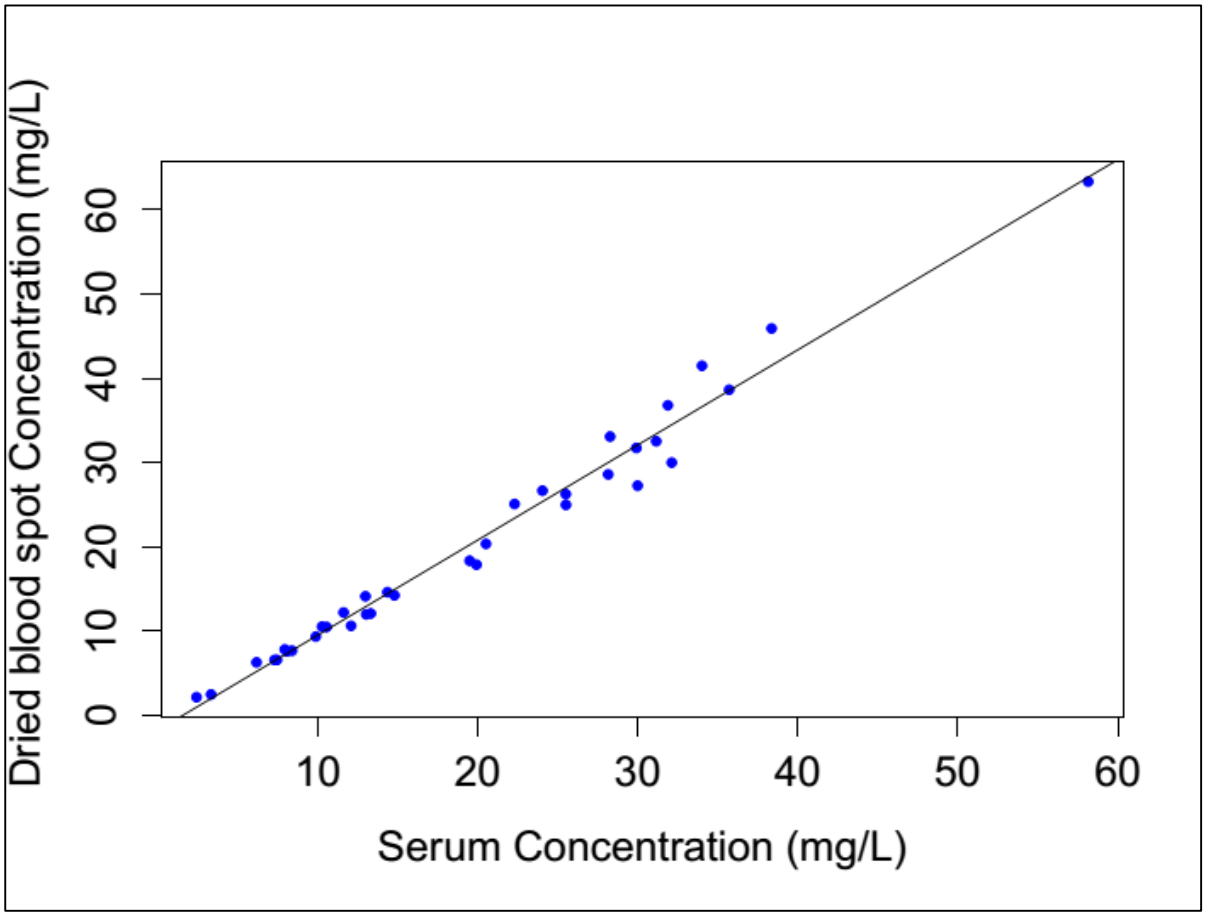
6: Regression plot and to determine the statistical regression using Spearman Rank Correlation Test:

* R script for the above (# signifies a comment):

```
attach(Final_pts)

# For regression plot:

plot(DBS.Conc.~Serum.Conc.,pch=20,col="blue",xlab="Serum Concentration (mg/L)",
ylab=" Dried blood spot Concentration (mg/L)",main="" )

# pch= 20 is a solid circle when plotted on a graph

x=Serum.Conc.

y=DBS.Conc.

abline(lm(y~x))   #lm stands for linear model
```

Regression plot of serum concentration versus corrected dried blood spot concentration.is shown in *Figure 31*.

Figure 31: Regression plot of serum concentration versus corrected dried blood spot concentration.



Using the Spearman rank correlation test, which is the preferred test for non-parametric data, the correlation of SC and CDBS concentration, was performed.

* R script for Spearman correlation (# signifies a comment):

```
attach(Final_pts)

cor(Serum.Conc.,DBS.Conc.,method="spearman")
```

Result: The Spearman rank correlation between the serum and the corrected dried blood concentration was 0.9878.

Spearman correlation sensitivity test result (after the removal of the outlier patient):

$\rho = 0.9867$.

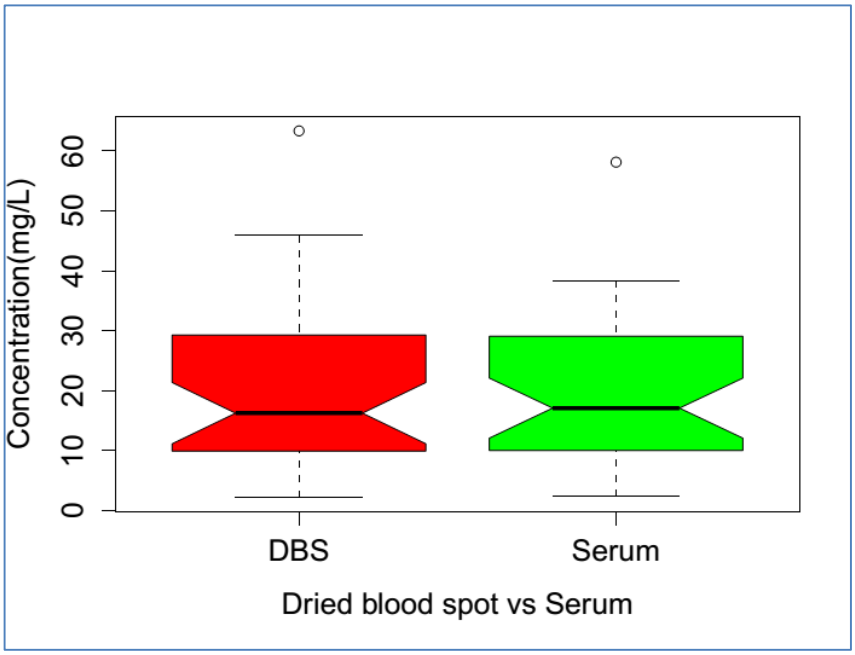
7: Box plots showing median concentration values and outlying data points

Data file (Final_group) configuration is shown in *table 6* in the appendix. Data must be grouped vertically as two groups (Serum and DBS) and a column of corresponding data in order for the labels to appear at the bottom of each of the groups.

* R script for the above (# signifies a comment):

```
attach(Final_group)      # Final_group is the csv file where the data is arranged
                         # vertically as two groups in one column
# Boxplot with the two (Serum Vs Corrected DBS concentration):
boxplot(Conc.~ Group,col=c("red","green"),notch=TRUE,main="",xlab="Dried
blood spot vs Serum",ylab="Concentration(mg/L)")
```

Figure 32: Box plot of Serum and Corrected dried blood spot concentration



Conclusion: The box plot shows that the medians of the two groups are similar and there is 1 outlier in both groups.

8: Paired sample Mann Whitney-U test

The paired sample Mann Whitney-U test confirmed that the null hypothesis (i.e. there is no difference between the serum and CDBS concentrations) should be retained.

The result is shown in *table 16:*

* R script for the above (# signifies a comment):

```
attach(Final_pts)

# Mann Whitney U test
wilcox.test(Serum.Conc.,DBS.Conc.,mu=0,alt="two.sided",conf.int=TRUE,
conf.level=0.95, paired=TRUE,exact=FALSE,correct=TRUE)
```

Table 16: Results of the Mann Whitney U test

Null hypothesis	Test	p-value	Decision
There is no difference between the serum concentration of PHB and that measured in the CDBS	Paired samples Mann Whitney - U test	0.3919	Retain the null hypothesis

Determination of relevance of spotting inconsistencies

1. The summary data of the patients used to determine the relevance of spotting inconsistencies (n=13) is shown in *Table 17.*

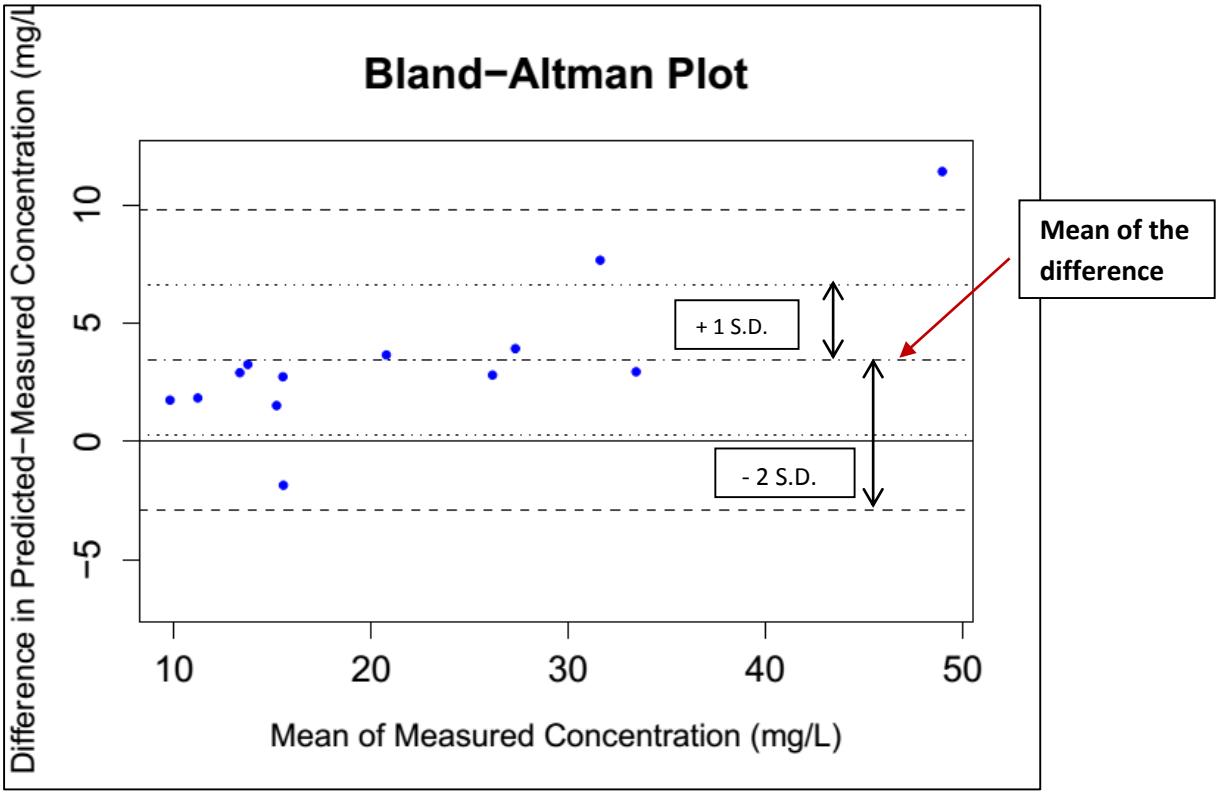
Table 17: Summary data of patients used to determine the relevance of spotting inconsistencies

I.D.	Serum.Conc.	DBS.Conc.	Difference between DBS & Serum	Mean
Mean	19.68	20.49	0.8103	20.09
Median	17.13	16.29	-0.035	16.72
Minimum	2.41	2.2	-2.69	2.3
Maximum	58.14	63.36	7.59	60
1st Quarter	10.15	10.24	-0.7	10.21
3rd Quarter	28.67	28.98	1.53	29.15

2. The imprecision and bias between the measured and predicted serum concentration was found to be 18.61% and 17.20% respectively.

3. The Bland Altman plot, *Figure 33*, shows that there is a large positive bias. The mean of the difference between the predicted and the measured serum concentration was 3.44 with a standard deviation of 3.17.

Figure 33: Bland Altman plot - spotting inconsistency (n=13)

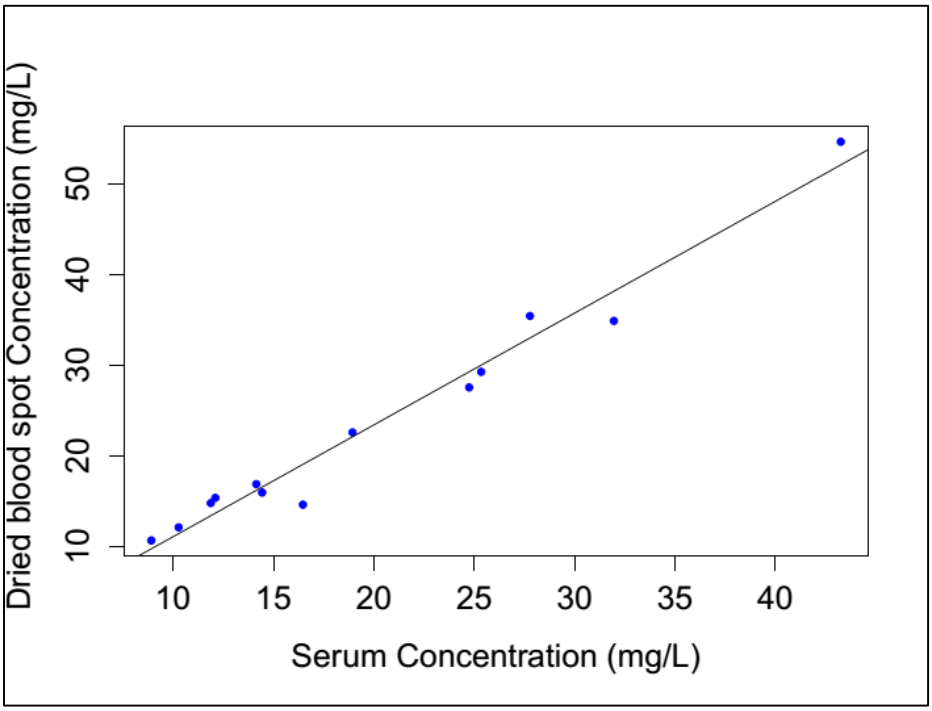


4. Regression plot and determination of statistical regression

The statistical regression determined using the Spearman correlation test was found to be 0.9340.

The regression plot is shown in *figure 34*.

Figure 34: Regression plot of serum vs corrected dried blood spot concentration in specimens used to determine the relevance of spotting inconsistencies



5. An independent Mann Whitney U test showed that there is a significant difference between the serum and the corrected dried blood spot concentrations in this group of patients. This is shown in *table 18.*

Table 18: Results of Mann Whitney-U test

Null hypothesis	**Test**	**p-value**	**Decision**
There is no difference between the serum concentration of PHB and that measured in the CDBS	Paired samples Mann Whitney U test	0.0033	The null hypothesis is discarded

Discussion

Phenobarbitone is a widely used antiepileptic used in the treatment of seizures (21). Therapeutic drug monitoring of phenobarbitone has been recommended for optimizing drug treatment (26,58). The conventional serum or plasma specimens used to monitor phenobarbitone concentrations pose the problems of requiring a cold chain facility for the specimens to be transported as well as increased risk of biohazard to handlers. Dried blood specimens provide a safer (69,75) and a cheaper alternative to small hospitals to send specimens for phenobarbitone monitoring.

The HPLC assays required for the measurement of phenobarbitone in serum and dried blood spot were both developed and validated in our laboratory. Both the assays were simple precipitation extractions and showed good sensitivity, specificity, reproducibility and precision. The calibration curve for serum was linear from 2.5 to 50 µg/ml. The intra-day and inter-day variation in the assay expressed as % CV was less than 2% and less than 4% respectively for concentrations across the calibration curve. The % CV in repeat injections was less than 3% and the lowest concentration that was quantified with a discrete peak and an area more than 5 times that of a blank specimen was 2.5 µg/ml. Since it is well known that phenobarbitone is stable in serum/ plasma for a period of upto 4 weeks at – 20 °C (81,82), we did not evaluate this.

The linearity of the calibration curve for dried blood spot ranged from 2.5 to 40 µg/ml. The intra-day and inter-day precision of the assay expressed as % CV was less than 9% and less than 7% respectively. The % CV in the repeatability of injections was less than 3% and the assay showed a good recovery of 89.2%. Since the local temperature varies from place to place and because the DBS specimens once spotted will be sent by mail for analysis to the TDM centres, we evaluated the stability of DBS specimens under varying temperatures. Stability was tested at laboratory temperatures (24 -30 °C) and simulated transport conditions

(35 – 40 °C). Transport was simulated by storing the papers in a car and transporting them over a period of 26 days. The results showed that the stability of the DBS specimens was retained for this period of 26 days when stored in both the laboratory and the car.

Since the protein saver #903 card, which is normally used for spotting the whole blood, is expensive and this assay is primarily aimed for use in rural hospitals and poorer population, we used the standard Whatman filter paper for the dried blood spot analysis. Because this paper is not standardized for width, the whole blood spot which contains a known volume of blood (20 µl), was cut out and used for extraction. Due to the disadvantages of the finger prick method (as mentioned in *pages 44 & 45*), we employed the second method of spotting blood using a calibrated micropipette.

This was an open label, prospective study where the serum and dried blood spot specimens of 36 patients who were on phenobarbitone, were analysed and the concentrations compared. The imprecision and bias between the measured and the predicted serum concentrations of phenobarbitone was found to be higher when the DBS concentration was uncorrected for PCV (UDBS), than when corrected for PCV (CDBS). Thus analysis was done with the CDBS concentrations.

The graphical and statistical methods demonstrated that the data in both the groups (serum concentration and CDBS concentration) were not normally distributed. Non-parametric tests were therefore used for analysis. The Spearman rank correlation test showed a good correlation between the concentration of phenobarbitone in serum and the corrected dried blood spot ($\rho = 0.9878$). A box plot of the two groups showed 1 outlier in each of the groups. This could be identified as patient XXXX. Sensitivity analysis was performed by removing the outlier and this showed that this patient had no significant effect on the data as the correlation was maintained ($\rho = 0.9867$). Our study showed a very good correlation between

the serum and the CDBS concentrations which agrees with La Marca et al (74). The Mann Whitney-U test confirmed that there was no difference between the measured concentration in serum and that in the dried blood spot (p = 0.3919). Since the therapeutic range of phenobarbitone has been established for serum/ plasma, it is necessary to determine a serum/plasma concentration. However, due to the Mann Whitney test confirming that there is no statistical difference between the two groups we can use the PHB concentration in the DBS as the predictive measure of PHB in the serum without having to employ an equation to relate the two. A possible reason for the agreement in the concentration of PHB between the two groups could be the low protein binding of the phenobarbitone (40 – 60%).

The Bland Altman plot showed a small overall positive bias. However, it does seem to indicate a trend which may have to be considered for concentrations above 30 µg/ml. The numbers of specimens in this study with concentrations over 30 µg/ml was only 7, out of which only 3 had a % difference ([(serum concn – CDBS concn)/serum] * 100) of more than 10% and only 1 more that 20%. In only 2 of the patients would this result have possibly caused an incorrect change in dose as the measured serum concentrations were 38.4 µg/ml and 34 µg/ml respectively with the corresponding CDBS concentrations of 45.9 µg/ml and 41.5 µg/ml. More work needs to be done, to determine if this is a true effect and if so what correction, if any, needs to be applied.

Spotting inconsistencies were seen during the spotting process when a different volume of blood remained in the tip after the blood had been delivered to the filter paper. When the degree to which these inconsistencies are important was evaluated, it became clear from the results (precision, bias and the Mann Whitney-U test) that there is a highly significant difference between the predicted and the measured serum concentrations (p=0.0033). The Bland Altman plot *(page 112)* also showed a much higher positive bias with the mean of the

difference between the predicted and measured serum concentrations as 3.44 ± 3.17. This highlights the importance of correctly spotting the specimens.

Limitations of the study/assay/DBS spotting technique

- The DBS assay had a long run time of 21 minutes since the drug – phenobarbitone had a long retention time. (RT= 19.343 minutes, under the final standardized HPLC conditions).

  Phenytoin and carbamazepine elutes even later than phenobarbitone under the final standardized HPLC conditions of both the serum and DBS assays. Hence, patients on these drugs had to be given a longer run time to prevent the carryover in the next run.
- The spotting of the DBS specimens requires technical precision. Hence, the personnel performing the DBS spotting have to be adequately trained.

Conclusion

The concentration of phenobarbitone in serum can be accurately predicted from that measured in DBS without the need of an equation. The dried blood spot is a valid alternative to using serum or plasma for the measurement of phenobarbitone concentration. Thus, a cheaper and a safer means for TDM of phenobarbitone can be made available to the smaller and rural hospitals in India.

More research needs to be carried out in evaluating the bias involved in interpreting CDBS concentrations above 30 μg/ml.

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Appendix

Date	Standards (µg/ml)	Ratio	Value (µg/ml)
4/4/2012	40	1.09	
	10	0.32	
	5	0.2	
	2.5	0.06	
	QC 1 (20)	0.6	21.7

Results of the stability of phenobarbitone in dried blood spot (upto 26 days) - Tables 1 & 2

No of days when specimens were checked	Standard curve (µg/ml) 11/04/2012	Ratio	Concentration (µg/ml)	Specimen ID	Spiked value	Lab Standards and QC (4/4/2012) against new standard curve		Car Standards (4/04/2012) against new standard curve	
						Ratio	Concentration	Ratio	Concentration
7 days (11/04/2012)	40	1.17		1	40	1.17	40.63	1.11	38.56
	10	0.24		2	10	0.3	10.63	0.29	10.29
	5	0.16		3	5	0.18	6.49	0.16	5.80
	2.5	0.08		4	2.5	0.07	2.7	0.07	2.70
	QC 2 (20)	0.58	20.28	5	20	0.57	19.94	-	-
	QC 2 against the standard curve of 4/4/2012		**19.44**						
	Lab QC (4/4/2012) aginst standard curve of 4/4/2012		**19.1**						
	Lab QC (4/4/2012) against the car standard curve (4/4/2012)		**20.63**						

No of days when specimens were analysed	Spiked values	Lab Standards (11/04/2012) Ratio	Concentration (µg/ml)	Spiked values	Car Standards (4/04/2012) Ratio	Concentration (µg/ml)
19 days 23/04/2012	40	1.09		40	1	
	10	0.23		10	0.26	
	5	0.11		5	0.13	
	2.5	0.02		2.5	0.06	
	QC (20)	-		QC (20)	-	
	Lab QC (4/04/2012) against the Lab standards (11/04/2012)	**0.56**	**21.61**	**Lab QC (4/04/2012) against the car standards (4/04/2012)**		**22.24**

Results of the stability of phenobarbitone in dried blood spot (upto 26 days) (cont) - Tables 3 & 4

No of days when specimens were checked	Standard curve (µg/ml) 30/04/2012	Ratio	Concentration (µg/ml)	Specimen ID	Spiked value	Lab standards and QC (4/04/2012) against new standard curve (30/04/2012)		Car standards (4/04/2012) against new standard curve (30/04/2012)	
						Ratio	Concentration	Ratio	Concentration
26 days (30/04/2012)	40	1.06		1	40	1.04	40	0.95	36.4
	10	0.28		2	10	0.28	9.6	0.3	10.4
	5	0.18		3	5	0.15	4.4	0.17	5.2
	2.5	-		4	2.5	0.05	0.4	0.08	1.6
	QC (20)	-		5	QC (20)	0.55	20.4	-	-
	Lab QC (4/04/2012) against the lab standards 4/04/2012	**0.55**	**20.85**						

Figure 1: Extraction of Phenobarbitone from DBS

Figure 2: Extraction of phenobarbitone from serum

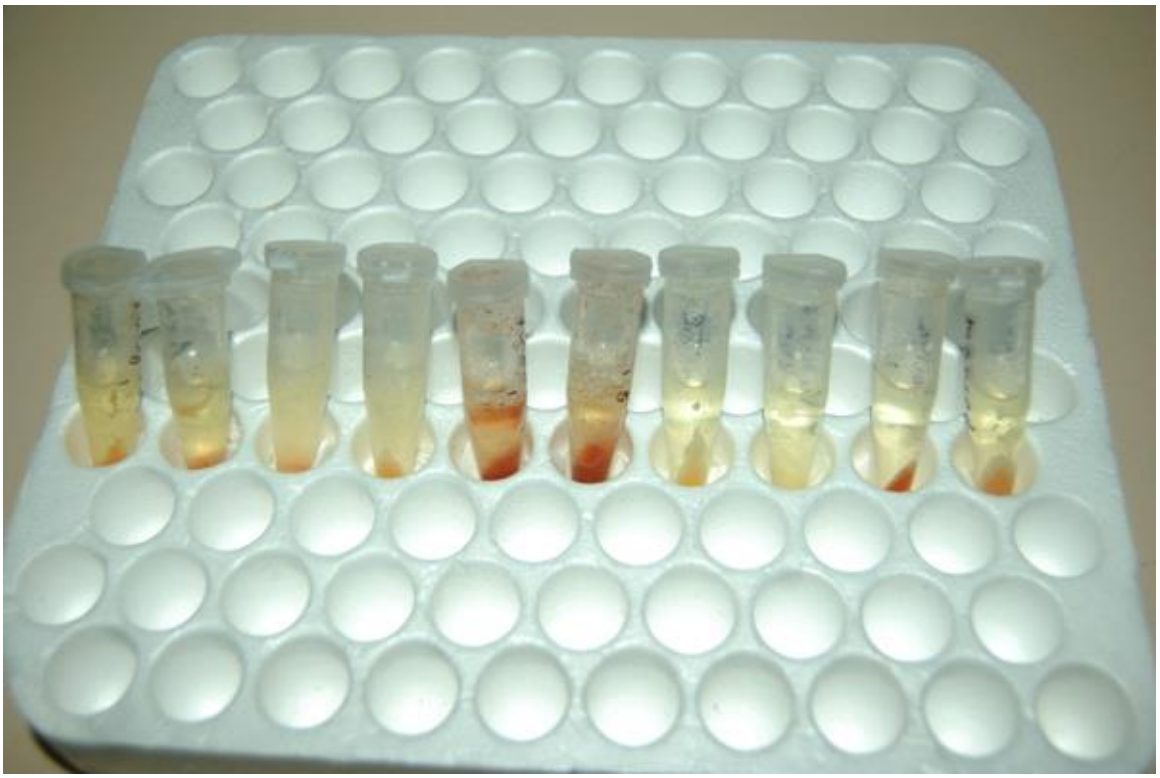

Table 5: *Format of CSV file data used for analysis using R program (for all statistical test except Box plot)*
File name – Final_pts

I.D.	Serum.Conc.	DBS.Conc.	Difference between DBS and serum conc.	Mean of the DBS and serum conc.
F1	31.14	32.56	1.42	31.85
F2	12.97	14.17	1.20	13.57
F3	9.87	9.39	-0.49	9.63

Table 6: *Format of CSV file data used for Box plot*

File name – Final_group

Group	Conc.
Serum	31.14
Serum	12.97
Serum	9.87
DBS	32.56
DBS	14.17
DBS	9.39